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Modulation of sulphur mustard cellular toxicity by ATP

A possible mechanism of action of sulphur mustard

P.M Lundy, R. Frew, C. Vair, M.G. Hamilton and T W. Sawyer
Defence Research Establishment Suffield

P. Nelson, W. Gong and L. Mi
Kinchyle Enterprises

Defence R&D Canada
External Contract Report
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February 2001

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Author

Dr. P.M. Lundy

Approved by

Dr. C.A. Boulet
Head/Chemical and Biological Defence Section

Approved for release by

Dr. R. Herring
Chair/DRES DRP

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Abstract

HD caused apoptosis and to a lesser degree, necrosis in a wide variety of cell types. As the concentration of HD was increased the proportion of necrotic cells became more predominant. HD produced a modest elevation of intracellular calcium levels in J774 and CHOK-1 cells, as well as in human skin keratinocytes, although this rise did not seem causally related to HD induced cell death. However, very recent evidence indicates that HD induced DNA fragmentation may not be directly related to cytotoxicity. HD also activated caspase-3 in CHOK-1 cells, but neither specific nor general caspase inhibitors nor proteosome inhibitors reduced HD cytotoxicity. These results suggest that the toxicity of HD is fundamentally different than apoptotic stimulae such as dexamethasone or X-irradiation, in which the apoptosis induced by both of these treatments can be reduced by the above mentioned protease inhibitors. Subtypes of the P2X receptors were identified in neuronal synaptosomes, J774 cells and CHOK-1 cells. In CHOK-1 cells $P2X_7$ receptor subtypes were identified and the specific $P2X_7$ inhibitor, oxidized ATP, was found effective in reducing HD induced DNA fragmentation and the appearance of soluable DNA. However, this treatment appeared only to shunt the mechanism of cell death from being predominantly apoptotic, to more necrotic in nature; however, the overall cytotoxicity remained unchanged.

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Introduction

Sulphur mustard (HD) may cause cell death as the result of a number of different inter-related biochemical events, several of which are currently being examined in this laboratory. The literature proposes that cell death, particularly apoptosis, but not excluding necrosis, results from the initiation of a cascade of events which may be initiated by the elevation of intracellular Ca^{2+} concentrations. Elevations of intracellular Ca^{2+} may in turn activate proteases, phospholipases and endonucleases, as well as other important intracellular regulators (for recent reviews see Kass and Orrenius, 1999; Nicotera and Orrenius, 1998). It has been proposed that the magnitude and duration of the Ca^{2+} elevation will determine the ultimate fate of the cell. With respect to survival, it appears likely that small, short periods of Ca^{2+} elevation will probably have no effect on cell viability, moderate or longer periods of elevation may lead to apoptosis, while large pulses, or longer duration of moderate elevation, would be expected to initiate necrosis.

Cellular calcium levels may be controlled through modulation of the influx of extracellular calcium by a variety of cell surface events. These include the activation of cell surface receptors which act through a second internal messenger, or as ligand gated ion channels Additionally, intracellular calcium can be controlled through modulation of cell surface voltage sensitive ion channels, especially in excitable tissue. Further, control of intracellular free calcium can also be effected through alteration of the sequestration of calcium (calcium binding proteins, mitochondria, etc.) which has entered the cell or which has been released from intracellular storage sites.

One group of cell surface receptors which help modulate the access of calcium to the interior of the cell are the P2X type of ATP receptors. Recent evidence suggests that extracellular ATP is a broad spectrum cytotoxic agent which promotes apoptosis by activation of distinct cell surface P2X purinoceptors (Burnstock, 1998). These receptors act as ionotropic channels, for example, to transport calcium through the cell membrane. This results in a variety of physiological effector responses such as muscle contraction, neurotransmitter release and of course, the activation of various enzymes, some of which mediate catalytic functions in the destructive process (Burnstock, 1998; Evans *et al.*, 1992; Kass and Orrenius, 1999). In addition to their physiological roles, at least two of the ATP receptors in this sub-family are also thought to mediate programmed cell death. They may also contribute to necrosis as well, since these processes may be more closely inter-related than previously thought (Raffray and Cohen, 1997).

The $P2X_1$ subtype of the P2X receptors has been proposed to initiate apoptosis following activation and has been shown to be upregulated in cells undergoing this type of cell death (Chvatchko *et al.*, 1996; Zambon *et al.*, 1994). This particular receptor has marked homology (Valera *et al.*, 1994; Brake *et al.*, 1994) to the product of the RP-2 gene, which has been shown to encode for apoptosis (Owens *et al.*, 1991). A second subtype of P2X receptor family, the $P2X_7$ receptor, is not only an ionotropic receptor which is permeable to ions such as calcium, but is also coupled to a non-specific membrane pore (Pizzo *et al.*, 1991). Following activation by ATP and its analogues, this pore opens to allow the entrance into the cytoplasm of large molecules up to about 700 daltons (Hickman *et al.*, 1996; Wiley *et al.*,

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1996). The literature is somewhat vague, but suggests that activation of this receptor and the entrance of the larger molecular weight species may initiate cell death, although it is not clear whether these events activate apoptosis, necrosis (colloidal osmotic lysis), or both types of cell death. It is believed that the cytotoxic activity of ATP is generally the result of the stimulation of one or both receptor types and that the type of toxicity induced, either apoptosis or necrosis, is dependent to some degree on the type of receptor present on the target cell.

HD is also a broad-spectrum cytotoxic agent, albeit more potent than ATP. However, the mechanism of cytotoxic action is not known (Papirmeister *et al.*, 1991). This cooperative agreement was predicated on a proposal that the cytotoxic action of HD was in some way related to the P2X receptors that mediate ATP induced cell death. The proposal was based on observations that these two cytotoxic agents markedly potentiated one another in eliciting a physiological response in smooth muscle preparations known to be mediated by the $P2X_1$ receptor. Since both $P2X_1$ and $P2X_7$ ATP receptors mediate cell death, we hypothesized that the $P2X_1$ receptor, and possibly the $P2X_7$ receptor, might also mediate some of the cytotoxic effects of HD (Lundy *et al.*, 1998, Hamilton *et al.*, 1998). Part of this report, therefore, describes attempts to identify the subtype of the P2X receptors on cells to which both ATP and HD are toxic. One of our priorities was to identify the types of ATP receptors on the cells which we have utilized as targets of HD induced cell death. Once these receptors were identified, manipulation of the activity or sensitivity of these receptor subtypes could be attempted in order to produce measurable changes in cellular viability following exposure to HD and ATP. For example, ATP receptor activity or sensitivity was assessed by utilizing selective ATP-blocking agents prior to exposure to the cytotoxic agents. Changes in intracellular calcium concentrations were measured by a variety of techniques and the role of ATP receptors acting as calcium pores in the membrane was examined. Other aspects of ATP receptor activity in cells containing $P2X_7$ receptors were investigated by measuring the uptake of dyes small enough to pass through the $P2X_7$ receptor (Michel *et al.*, 1998; 1999), or by utilizing non-ionic buffers which have recently been shown to enhance ATP receptor activity (Michel *et al.*, 1999). These studies were carried out in an attempt to correlate the effects of receptor activity with the cytotoxicity of ATP and HD.

An additional reason that we chose to study intracellular calcium was to provide a link between the activation of cell surface ATP ionotropic channels and cytotoxic enzymes such as the caspases, which have been implicated in the calcium dependent initiation of apoptosis (Cohen, 1997; Hengartner, 2000; Nicholson and Thornberry, 1997; Kidd, 1998). We have reported that HD does indeed elevate calcium levels in a variety of tissues (see addenda) and we are now examining the hypothesis that HD activates caspases critical to the initiation of cell death subsequent to the elevation of calcium. The role of caspase (or proteasome) activation in the genesis of HD-induced cell death forms another part of this report. In summary, we outline here our efforts to examine the toxicity of HD and to test the hypothesis that it produces apoptosis or necrosis through activation of P2X types of ATP receptors, inducing intracellular calcium elevation, resulting in protease or endonuclease activation and subsequent cell death.

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Methods

Cell Culture and Cytotoxicity Assays

Human Skin Keratinocyte Culture Primary cultures of human skin keratinocytes were prepared from neonatal foreskins. Tissue was obtained on the day of circumcision and incubated at 4°C for 24 h in 25 U/ml dispase (Collaborative Research, Bedford, MA). The epidermis was removed and the cells segregated by a further 5 min incubation in 0.25% trypsin at 37°C. The trypsin was deactivated by the addition of 20% serum in medium and the cell suspension was centrifuged. The cell pellet was resuspended in Keratinocyte Serum Free Medium (KSFM, Gibco BRL, Grand Island, NY) and filtered through 70 µm nylon mesh. 75 cm^2 flasks were seeded at 5 x 10^5 cells/8 ml KSFM supplemented with gentamicin (50 µg/ml) and Fungizone (0.25 µg/ml) and incubated in a 37°C humidified incubator in a 5% CO_2/95% air atmosphere. Cultures were re-fed every 2-4 days, as required. First passage cultures were seeded from log growth primary cultures at a density of 1,000 cells/well in 96-well Costar multiwell plates or in 35 mm culture dishes at 50,000 cells/dish.

Chick Embryo Neuron Culture Culture of chick embryo neurons were prepared as previously described (Weiss and Sawyer, 1993) and routinely plated at a density of 100,000 cells/well in 96 well titer plates coated with 0.1 ml/well of 12.5 µg/ml polylysine in water. The cells were seeded in 0.1 ml of 5% horse serum in mMEM supplemented with streptomycin (100 µg/ml), penicillin (100 IU/ml) and Fungizone (2.5 µg/ml).

CHO-K1 and J774 Cell Line Culture Seed cultures of both cell lines were obtained from the American Type Culture Collection. The cells were grown in 10% FCS in DMEM supplemented with streptomycin (100 µg/ml) and penicillin (100 IU/ml) and the medium was changed twice a week. Subconfluent cultures were passaged (1:3) into 75 cm^2 culture flasks or into 96 well titerplates at a density of 2000 (CHO-K1) or 4000 (J774) cells/well.

Chemical Treatment and Cytotoxicity Studies On the day of chemical treatment the cultures were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0.25% ethanol (v/v). The viability of HD-exposed cultures was determined at 24 or 48 h. In experiments which assessed the effects of BAPTA-AM, ionomycin or thapsigargin on HD toxicity in human keratinocytes, the compounds were dissolved in ethanol or DMSO (BAPTA-AM) and administered to the cultures 1 h prior to HD treatment. In studies where external calcium was also varied, 1 h before drug treatment, the cultures were aspirated, rinsed twice with 200 µl of phosphate buffered saline and then re-fed with calcium-free medium that had been adjusted to the desired calcium concentration with calcium chloride. In these experiments, Fura-2 was not used to quantitate the external calcium and therefore these values represent the *nominal* calcium concentrations. In all experiments, test drugs were left in the cultures for the full test period. The test drug vehicles (ionomycin; 0.046% ethanol, thapsigargin; 0.1% ethanol, BAPTA-AM; 0.15% DMSO) had no effect on the viability of the cultures, even in combination with the ethanol used as the HD vehicle. To assess cytotoxicity, alamarBlue (AccuMed International Inc., Westlake, OH) was added (10%, v/v) and the cultures were allowed to incubate with the dye for the last 2-5 h of

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the treatment time period. This assay is based on the reduction of a dye by viable cells to a coloured species which can be measured by absorbence or fluorescence, and has also been found to yield similar results compared to a number of more commonly used dyes and indicators (Fields and Lancaster, 1993). The absorbences (570 nm - 600 nm) were then read on a Themomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing 6 wells per data point. All values represent data obtained from at least 3 separate experiments. Sulphur mustard was prepared by the Hazard Avoidance Section, Defence Research Establishment Suffield, at greater than 99% purity.

Calcium Measurements

Fura-2 Studies Studies to assess the effect of HD on Fura fluorescence were performed using the cell impermeant pentapotassium salt of Fura-2 (0.4 μM) in high (1 mM, pH 7.4) and relatively low (225 nM, pH 4.5) Ca^{2+} buffer solutions (Molecular Probes, OR). Fluorescence of Fura-2 was monitored with a Delta-Scan 1 dual excitation/emission fluorometer with front-surface optics sample compartments (model D105; Photon Technology International Inc., Princeton, NJ). The output from the xenon lamp was directed to two excitation monochromators with wavelengths set at 340 and 380 nm (5 nm bandpass), respectively, using a chopper wheel. Output from the excitation monochromators was focused on a 1 cm^2 quartz cuvette by a fibre-optic cable and the fluorescence collected through an emission monochromator at 510 nm (5 nm bandpass) using a photomultiplier tube. Cellular experiments were performed after loading the cells with the membrane permeant form (Fura-2 AM) of the calcium sensitive dye Fura-2 (Grynkiewicz *et al.*, 1985; McDonough and Button, 1989). The cell suspension was rinsed three times with HEPES buffered Hank's balanced salt solution (pH 7.4, 1 mM nominal Ca^{2+} concentration). The cells were then incubated in the dark with 3 μM Fura-2 AM in DMSO/pluronic-127 for 45 min at room temperature. After incubation, the buffer was changed to buffer containing no Fura-2 AM and allowed to equilibrate for at least 30 min prior to recording fluorescence signals. The cell suspension (2.4 ml) was placed in a quartz cuvette and measurements were made with continuous stirring in a temperature-controlled cuvette holder at 37°C. HD was added to the cuvettes and the signal re-acquired after a delay of 1 min to eliminate an artifact due to the dissolution of the HD in the buffer. Signals were background subtracted using Fura-2 free cells for autofluorescence and, where appropriate, solvent addition.

Ca^{2+} Influx Studies Ca^{2+} influx was carried out according to the method of Blaustein (1975) with modifications (Lundy *et al.*, 1991). Synaptosomes were incubated at 30°C in the presence or absence of antagonist drugs for 15 min, at which time a 100 μl aliquot of the synaptosomal suspension was quickly injected into an equal volume of resting buffer (5 mM K^+), depolarizing buffer (25 mM K^+, final concentration), or resting buffer to which ATP or its analogues had been added (final concentration 1 mM), and all containing 0.5 μCi $^{45}Ca^{2+}$ (New England Nuclear, Boston, MA). Basal and K^+ stimulated Ca^{2+} influx were allowed to proceed for 3 sec, and basal and nucleotide-stimulated for 10 to 90 sec, at which time it was stopped by rapid dilution of the buffer with 4 ml ice-cold Ca^{2+}-free buffer containing 4 mM EGTA. Each suspension was rapidly filtered under vacuum through 0.45 μm membrane filters (Gelman Science) using a filtration apparatus (Hoeffer Scientific, San Francisco, CA). The membrane filters were washed twice with 5 ml resting buffer containing excess Ca^{2+}.

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Membrane filters were allowed to dry, placed in scintillation cocktail and counted on a Wallac 1500 scintillation counter. Assays were carried out in triplicate. Basal influx was subtracted from K^+ or nucleotide-stimulated influx and results expressed as Ca^{2+} influx (nMole per mg tissue). Experimental protocols were designed so that the effects of ATP, and five nucleotide analogues, could be examined on each synaptosomal preparation. In addition, K^+-evoked Ca^{2+} influx was included to assess the viability of each preparation.

In studies that investigated the actions of monovalent and divalent cations on BzATP-evoked Ca^{2+} influx and intracellular calcium, a NaCl buffer, and a NaCl-free, Mg^{2+}-free buffer, with reduced Ca^{2+}, supplemented with iso-osmotic sucrose were used. The NaCl buffer contained (in mM): NaCl 132, $CaCl_2$ 1.5, $MgCl_2$, KCl 5, D-Glucose 20, HEPES 20, with the pH adjusted to 7.4. The cation-free buffer (Michel *et al.*, 1999) contained sucrose 280, $CaCl_2$ 0.5, KCl 5, D-Glucose 10, HEPES 10, N-methyl-D-glucamine 5 with the pH adjusted to 7.4.

Apoptosis Detection Techniques

Genomic DNA Analysis The detection of degraded DNA products was carried out essentially as described by Herrmann *et al.* (1994). Cells were lysed in 50 µl of lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, and 1% Nonidet P-40) for 30 sec. After centrifugation at 2,000xg for 5 min at room temperature, the fragmented DNA in the supernatant was collected. After repeating the centrifugation step once, the supernatant was further treated at 56°C for 2 h with RNase A (5 mg/ml) and SDS (1%), followed by digestion with proteinase K (2.5 mg/ml) at 37°C for 2 h. DNA was precipitated and electrophoresed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and visualized under UV light. A 100-bp DNA ladder (Bio-Rad, Mississauga, Ontario, Canada) was used as a size marker.

TUNEL Reaction For *in situ* nick end labeling (TUNEL reaction), cells were plated onto 25 mm Thermonox plastic culture coverslips and allowed to grow to subconfluency prior to experimental use. After HD treatment (typically 4-6 h), the medium was removed and the coverslips were washed twice with PBS, and then fixed with 4% paraformaldehyde for 30 min. The cells were permeabilized for 2 min with 0.1% triton X-100, 0.1% sodium citrate and after air drying, 50 µl of TUNEL reaction mixture (Roche Molecular Biochemicals, Laval, Quebec, Canada) was added so as to cover all cells. The coverslips were incubated at 37°C in a humidified chamber for 60 min and then rinsed 3 times with PBS prior to analysis by fluorescence microscopy.

Annexin V and Propidium Iodide Staining In the early phase of apoptosis, phosphatidylserine is translocated from the inner part of the membrane to the outer layer. Fluorescein-conjugated Annexin V specifically binds to the phosphatidylserine on the outer leaflet of the cell membrane. Propidium iodide was used in combination with Annexin V to differentiate apoptotic from necrotic cells. Cells were cultured in 24 well plates to subconfluency prior to experimental use. After HD treatment (typically 4-6 h) the treatment medium was removed and the cells washed twice with PBS. The cultures were then treated with 50 µl of Annexin V and propidium iodide staining-solution (Roche Molecular Biochemicals, Laval, Quebec, Canada) for 10 min at room temperature and analysed under a fluorescence microscope. Annexin-positive and propidium iodide-negative cells were defined as apoptotic, whereas necrotic cells were positive for both labels.

Soluble DNA Soluble DNA was measured by the method described by Cui *et al.* (1994) with modifications. The cells were grown in 24 well plates and log phase growth cultures were radiolabeled by incubation with [^{3}H]thymidine (1 μCi/ml, Amersham Canada Ltd., Oakville, Ontario, Canada) overnight at 37°C in a humidified 5% CO_2 incubator. The medium containing [^{3}H]thymidine was then removed and the cells rinsed once with PBS. After drug treatment (3-6 h), aliquots of the culture medium (1 ml/well, part A) were saved for radioactivity measurement and the cells were then lysed in 0.5 ml TET (10 mM TRIS-HCl pH 7.5, 2 mM EDTA, 0.2% Triton X-100) at 4°C for 30 min. The cell-lysate was then centrifuged (22 min, 12,000 x*g*) and the resulting supernatant (part B) was removed and counted. The lysate pellet (part C) was solublilized with 1N NaOH (0.2 ml/well) and counted. The experiments were performed in triplicate. Soluble DNA (percentage) was calculated according to the following formula:

Part A (dpm) + Part B (dpm)/[Part A (dpm) + Part B (dpm) + Part C (dpm)] x 100.

Morphological Observations Cell cultures were grown to subconfluency in 24 well plates prior to experimentation. After HD treatment (4-6 h), the cells were washed with PBS and then stained with 10 μl dye mix (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS) as previously described (Duke and Cohen, 1992). The cells were then visualized and scored using fluorescence microscopy. A minimum of 200 cells was visualized, recording the incidence of each of the following four cellular states: i) viable cells with normal nuclei (VN; bright green chromatic with organized structure); ii) viable cells with apoptotic nuclei (VA; bright green chromatic which is highly condensed or fragmented); iii) nonviable cells with normal nuclei (NVN; bright orange chromatic with organized structure); and iv) nonviable cells with apoptotic nuclei (NVA; bright orange chromatic which is highly condensed or fragmented). The percentages of apoptotic and necrotic cells were then calculated according to the formula:

% apoptotic cells = (VA + NVA)/(VN + VA + NVN + NVA) x 100.

% necrotic cells = NVN/(VN + VA + NVN + NVA) x 100.

Detection of mRNA for $P2X_1$ and $P2X_7$ Receptors Total RNA was extracted from CHO-K1 cells according to a guanidium isothiocyanate protocol as previously described (Ghahary *et al.*, 1994). cDNA (RT-cDNA) was made from total RNA by using a cDNA kit (Boehringer Mannheim, Quebec, Canada) and following the manufacturer's instructions. PCR was carried out in an automatic thermal cycler (PTC-2000 peltier thermal cycler, MJ Research, MA). Sense primers with the sequence of 5'-AGAGGCACTACTACAAGCAGAA-3' (for $P2X_1$) and 5'-AGGAGCCCCTTATCAGCTCT-3' (for $P2X_7$), and antisense primers with 5'-GGTAAGGCTGTGGGAAAGA-3' (for $P2X_1$) and 5'-CATTGGTGTACTTGTCGTCC-3' (for $P2X_7$) were used to amplify the cDNA specific for the $P2X_1$ and $P2X_7$ receptor. The reaction mixture contained 1.5 M $MgCl_2$, 200 μM of dATP, dCTP, dGTP, and dTTP, 20 pmol of $P2X_1$ sense and antisense primers or 50 pmol of $P2X_7$ sense and antisense primers, 1.5 units Taq DNA polymerase for $P2X_7$, and 0.25 of the RT-cDNA (5 μl) in a final volume of 50 μl. The PCR conditions were 60 sec of denaturing at 94, 60 sec of annealing at 55, and 90 sec of extension at 72 for 35 cycles with an initial denaturing cycle at 94 for 3 min and a final extension cycle at 72 for 5 min. The amplified products were analyzed on a 1.2% agarose (Gibco BRL, Grand Island, NY) gel containing 0.5 μg/ml ethidium bromide. The size of the PCR product was judged by using 100 base pair (bp) DNA molecular weight markers as references (Boehringer Mannheim, Quebec, Canada).

HD Induced Cell Death

Summary

In the first annual report we showed the concentration dependant cytotoxic effects of HD in a variety of cultured tissues. This included the calculation of the LC_{50} for HD in those tissues examined at that time (keratinocytes, neurons, CHO-K1 cells, J774 cells and thymocytes) (Fig. 1, first report). We also reported our initial efforts to characterize HD induced apoptotic cell death in different cell types. In the present report, we have completed these studies and characterized HD induced cytotoxicity in four cell types; neurons, CHO-K1 and J774 cells, and keratinocytes. The cell death in all cases was due to both apoptosis and necrosis. The former was the predominant type of cell death relative to necrosis, particularly at the lower HD levels examined. It was necessary to confirm the presence of apoptosis using a battery of apoptotic biochemical markers, since no one of these tests can be taken as proof of apoptosis (Renvoize *et al.*, 1998). Each cell type exposed to HD at predetermined concentrations was examined by the apoptotic markers outlined in Table 1. A small proportion of this data was presented in last year's report. However, for the sake of completeness and in order to present what is a consistent comprehensive picture of HD induced cell death, some data are repeated here.

***Table 1.** HD-induced apoptotic cell death in vitro*

	J774	CHO-K1	NEURONS	KERATINOCYTES
Annexin V	✓	✓	✓	✓
TUNEL	✓	✓	✓	✓
DNA Ladders	✓	✓	✓	✓
Soluble DNA	✓	✓	N/A	✓
Morphology	✓	✓	✓	✓

The effects of HD on soluble DNA levels were not assessed in chick embryo neurons since this assay requires proliferating cells. Except for this assay, HD induced a concentration response for all assays in all cell types.

Results

Figure 1 shows a representative concentration response curve obtained for each of the different test cell types when they were treated with HD. The macrophage-derived cell line J774 was much more sensitive than the CHO-K1 cells to the effects of HD, with LC_{50} values typically within the 50-75 μM range, compared to a LC_{50} range of 250-350 μM for the CHO-K1 and skin cells. Both keratinocytes and neurons showed differential sensitivity to HD, depending on the length of time they had been in culture. In contrast to keratinocytes, which became markedly more resistant to HD as they achieved confluency, neuronal cells became

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more sensitive to the toxicity of HD as they matured. All studies of apoptosis were carried out with just-confluent cultures of the cell lines, with immature neurons (one day *in vitro*) and with proliferating cultures of human keratinocytes (three to four days *in vitro*). HD-induced fragmentation of DNA was visualized using agarose gel electrophoresis and Fig. 2a-c shows the typical ladder patterns obtained when neurons (a), J774 cells (b), and CHO-K1 cells (c) were treated with HD. Ladder patterns of fragmented DNA were also observed with keratinocytes treated with HD. However, this type of fragmentation was not obtained consistently, and when seeen, was observed as an intensification of the faint laddering of DNA obtained from vehicle-treated control cells. Fragmentation was also assessed quantitatively by using a soluble DNA assay utilizing tritiated thymidine. With the exception of neurons (which could not be labeled), HD induced a concentration-dependent increase in fragmentation in every cell type, which appeared to plateau at the higher HD concentrations (Fig. 3a-c).

Figure 4 illustrates examples of the results obtained following the assay of fragmented DNA using the TUNEL reaction and fluorescent (Fig. 4a-c) or light microscopic (Fig. 4d-f) detection. In CHO-K1 cells, apoptotic nuclei are stained with fluorescein, and clumping chromatin can easily be discerned as intensely dyed inclusions. In human keratinocytes, light microscopy is used and the brown product of the peroxidase reaction indicates apoptotic nuclei. This assay lends itself well to quantitation and Fig. 5a-d shows that the number of apoptotic cells (as a percent of total cell number) increases with increasing HD concentration in all cell types. Figure 6a-c shows an example of the effects of HD on J774 cells when assayed morphologically. Control cells show normal green nuclei while HD induced the appearance of apoptotic nuclei (bright yellow condensed or fragmented chromatin). At higher HD concentrations, the cell membrane loses its integrity and ethidium bromide enters the cell, staining the chromatin orange. Figure 7a-d shows the quantitation of both apoptotic and necrotic cells in the different cell types using this assay. Generally, the percentage of apoptotic cells increases with increasing HD concentration. At higher HD concentrations, the percentage of cells that are necrotic becomes more predominant. Figure 8a-c shows examples of Annexin V binding to apoptotic CHO-K1 cells. Control cells are generally negative. At low HD concentrations, the fluorescein-labeled phospholipid-binding proteins recognize the externalized phosphotidylserine residues that are a hallmark of early stage apoptotic cell death and stain the cells green. As the concentration of HD becomes higher, the cell membranes of necrotic cells lose integrity and increasing numbers of cells also become stained with propidium iodide (red/brown colour). Similar concentration responses were also observed with the other three cell types (data not shown). This assay is not generally quantitated in our laboratory.

Discussion

Many techniques are now available with which to measure apoptosis, including detection of cell surface proteins (Annexin V), assessment of membrane integrity using various dye exclusion tests (trypan blue, ethidium bromide, propidium iodide, acridine orange), morphological assessments using light or electron microscopy, measurements of DNA fragmentation using radiological (soluble DNA), electrophoretic (DNA ladders) or immunological (TUNEL) means and molecular biological methods to measure the regulation of a variety of genes associated with apoptotic cell death. In many cases, not all endpoints

normally associated with apoptotic cell death may be induced by a specific agent or conversely, an endpoint normally associated with apoptosis will be detected in cells undergoing necrotic cell death. Since the nature of apoptotic cell death is often dependent on the target tissue and the inducing agent, assessment of a battery of endpoints is preferable when investigating mechanisms of cell death (Renvoize *et al.*, 1998).

A number of recent studies have shown that HD induces several of the hallmarks of apoptotic cell death in cell culture. In one of the earliest works, Dabrowska and coworkers (1996) showed, using gel electophoresis of DNA, as well as morphological criteria, that endothelial cells were induced to undergo an exclusively apoptotic cell death at low HD concentrations (< 250 μM), while at higher HD concentrations (> 500 μM), cells were undergoing both apoptosis and necrosis to an equal extent. Similar results have also been found in Hela cells (Sun *et al.*, 1998). Other laboratories (Michaelson, 2000; Meier and Millard, 1998) have used DNA gel electrophoresis exclusively to show that HD caused immune cells (thymocytes and lymphocytes) to degrade their DNA into the "ladder" patterns that have become the most prevalent endpoint used in apoptosis studies. Michaelson (2000) further showed that DNA fragmentation in thymocytes exposed to HD was time dependent, with initial formation of large fragments (50 -700 kilobasepairs) followed several hours later by further degradation to the internucleosomal ladder of oligomers of ~ 180 base pairs. Rosenthal and coworkers (1998) investigated the effects of HD on human skin keratinocyte gene expression and found that two genes intimately linked to apoptotic cell death were affected. Thus, p53 was induced while the expression of Bcl-2 was suppressed. In addition, they showed that HD induced caspase-3 activation and poly (ADP-ribose) polymerase cleavage, both indicators of apoptotic cell death.

The present studies expand and complement previous work and confirm that HD induces several different hallmarks of apoptotic cell death in cell culture. Furthermore, the selection of cell types in this study shows that HD exerts cytotoxicity in a remarkably conserved fashion. Thus, HD induces apoptotic cell death as assessed using morphology (acridine orange/propidium iodide), DNA fragmentation (DNA ladders, TUNEL assay, soluble DNA) and cell surface markers (Annexin V) in cells derived from different species (mouse, hamster, chicken, human), and from different organ/tissues (ovary epithelial, macrophage, brain neuron, skin keratinocyte), using different cell culture types (immortalized cell lines versus primary or first passage culture). That HD exerts its toxicity in such a conserved fashion *in vitro* strengthens the likelihood that similar mechanisms of cell death are also operative *in vivo*, a notion supported by recent findings that HD also induces several characteristics of apoptotic cell death in HD exposed pig skin (Smith *et al.*, 1997) and in hairless guinea pig skin (unpublished observations, this laboratory).

Although the different cascades of events that lead to apoptotic cell death are not fully understood it is clear that they can be regulated by a number of different biochemical determinants, including nitric oxide (NO), reactive oxygen species (ROS), caspase activation and intracellular calcium levels. Modulation of these endpoints offers the possibility that the prevention or treatment of HD induced apoptosis may be possible. Nitric oxide has received an enormous amount of research attention due not only to its myriad physiological functions, but also because it has been implicated in a number of disease states and pathologies when produced in excessive amounts. It has been suggested that NO and its ability to form an array of reactive species (NO^+, $NO^{\cdot}$, NO^-, $ONOO^-$) can induce apoptosis by destabilizing

mitochondria with subsequent intracellular calcium imbalance (Brune *et al.*, 1999; Richter, 1998). Efforts have been made to link the toxicity of HD with its effects on the activity of nitric oxide synthase (NOS) and indeed, several inhibitors of this enzyme have been shown to have marked protective efficacy against the toxic effects of HD in both neuronal and keratinocyte culture (Sawyer, 1998a; b; 1999; Sawyer and Risk, 2000, Sawyer *et al.*, 1996; 1998). However, the protection obtained appeared to be related to the chemical structure of arginine and these studies showed that NO did not play a role in either the toxicity of HD or in the protective efficacy of the drugs used.

The induction of apoptosis due to oxidative stress, either through generation of ROS or through depletion of intracellular antioxidant levels ie., glutathione (GSH) is also supported by an enormous amount of data (Fawthrop *et al.*, 1991; Hall, 1999), and efforts have been expended to link the cytotoxic effects of HD with the oxidative state of the target cells. Although it has been shown that intracellular GSH levels do decline following HD exposure, only very limited success has been achieved when intracellular GSH levels are boosted by pretreatment with GSH precursors such as N-acetylcysteine (NAC) (Gross *et al.*, 1993) or other cysteine ester GSH precursors (Wilde and Upshall, 1994). Interestingly, pretreatment of endothelial cells with NAC was shown to prevent apoptotic features of cell death but not those of necrosis (Dabrowska *et al.*, 1996). However, these studies were only carried out to six hours, a time frame that is unlikely to be reflective of the full expression time of HD toxicity and it is unclear what effect NAC would have on HD toxicity after longer time intervals. L-Thiocitrulline (Sawyer *et al.*, 1998; Sawyer and Risk, 2000) and thiourea (unpublished observations) have been shown to have excellent protective efficacy against HD cytotoxicity in both human skin keratinocytes and chick embryo neurons. These compounds do not exert their protective effects through NOS inhibition or by chemically reacting with HD, and their strictly prophylactic action does not suggest that they are interfering with the ongoing production of HD induced ROS. However, it is possible that the reducing activity of the thio-amino moiety of these molecules may lend itself to chemically reacting with a unique, but short-lived toxic ROS species within the lipid bilayer of the cell membrane. We are currently investigating this avenue of research.

In summary, HD induces a uniform pattern of cellular responses that are indicative of apoptosis over a broad spectrum of cell types. The remarkably conserved fashion in which HD induces apoptotic cell death indicates that strategies based on interfering with these pathways may be able to prevent or treat HD toxicity.

The Role of Intracellular Calcium in HD Induced Cell Death

Summary

Increased intracellular calcium levels have often been implicated as a possible triggering event in the initiation of apoptosis from a variety of toxic stimulae (Dowd, 1995; Orrenius *et al.*, 1989). Previous hypotheses concerning mechanisms of HD toxicity, including our own suggestions, have implicated increased intracellular Ca^{2+} levels as being causative in the activation of various proteases and endonucleases, leading to apoptotic events such as DNA fragmentation.

In our first annual report, we demonstrated that intracellular free calcium levels became elevated when keratinocytes, as well as the J774 and CHO-K1 cell lines were treated with HD. We suggested at that time that one of the goals of our second year of study was to demonstrate whether a cause/effect relationship could be established between the rise in intracellular calcium and the production of cell death. To this end, we manipulated the intracellular free calcium levels by using treatments which affected intracellular free Ca^{2+} levels in a reproducible fashion. Prior to exposing keratinocytes to HD, cells were exposed to 1) BAPTA-AM, to complex intracellular Ca^{2+}, 2) thapsigargin to deplete intracellular free Ca^{2+}, or 3) nominal Ca^{2+} free medium to reduce the possibility of Ca^{2+} influxes. The initial results with keratinocyte were somewhat surprising, since none of the Ca^{2+} manipulations produced any measurable effects on HD induced toxicity as measured by the metabolic viability indicator dye alamarBlue. Further studies were carried out in J774 cells and similar results were again obtained. These studies were then extended to include the effects of calcium modulation on HD induced apoptosis. Once again, no effects were observed on HD induced DNA fragmentation or apoptotic morphology. These results lead to the conclusion (see Annexes) that calcium does not play a role in HD induced toxicity.

Results

Figure 9 shows that the Ca^{2+} ATPase inhibitor, thapsigargin and the calcium chelator BAPTA-AM had no effect on the toxicity of HD in human skin keratinocytes. Figure 10 depicts the effect of HD on intracellular calcium levels in J774 and CHO-K1 cells. Intracellular calcium levels became elevated soon after HD exposure. Further studies were carried out in J774 cells to ascertain the role of calcium in HD induced apoptotic cell death. Figures 11 and 12 show that neither thapsigargin nor BAPTA-AM had any effect on HD induced DNA fragmentation as measured by soluble DNA (Fig. 11) or DNA ladders (Fig. 12). In addition, nominal zero external calcium concentrations (after BAPTA rinses of the cells to chelate residual calcium) did not effect HD induced DNA fragmentation (Fig. 11,12). Similar results were found when the morphology of the cells was assessed. No pretreatment had any significant affect on HD induced cell death as assessed using acridine orange and ethidium bromide to visualize the cellular toxicity (Fig. 13).

Discussion

The perturbation of intracellular calcium has been implicated in both apoptotic and necrotic cell death (Fawthrop *et al.*, 1991; Kass and Orrenius, 1999, Nicotera and Orrenius, 1998; Orrenius, 1985; Orrenius and Nicotera, 1987; Orrenius *et al.*, 1988) and HD has been shown by several groups to elevate intracellular calcium levels (Hamilton *et al.*, 1998; Hua *et al.*, 1993; Mol, 1994; Mol and Smith, 1996; Ray *et al.*, 1994; 1995), apparently through release from intracellular stores. Ray and coworkers have consistently reported that BAPTA-AM is protective (Ray *et al.*, 1996; 1997; 1998) and in recent studies they have also shown that BAPTA-AM, as well as the calmodulin inhibitor W-7, were effective in preventing several aspects of apoptotic cell death (Rosenthal *et al.*, 1998). In efforts to further investigate the role of calcium in HD induced toxicity, we attempted to reduce toxicity by modulating calcium levels in human skin keratinocytes and were not able to demonstrate any effects using nominal zero calcium medium concentrations, the Ca^{2+}-ATPase inhibitor thapsigargin or the intracellular calcium chelator BAPTA-AM (Sawyer and Hamilton, 2000). The reasons for the disparity in our results versus those of Ray and coworkers were unclear, although it was possible that perhaps the metabolic dye indicator that we used in these studies (alamarBlue) was not as sensitive an assay for toxicity as those used by the other laboratory. Accordingly, we carried out studies using J774 cells and measured HD induced DNA fragmentation as measured by DNA ladders and soluble DNA. In both assays, HD induced the DNA fragmentation characteristic of apoptotic cell death. However, neither nominal external calcium concentrations, nor thapsigargin, nor BAPTA-AM altered the degree, or the characteristics of fragmentation. Furthermore, the apoptotic morphology induced by HD was unchanged by these pretreatments.

To date our results do not support the conclusion that the elevation in intracellular calcium levels induced by HD in different cell types, is causal or involved in the resultant toxicity. However, very recent experiments in human keratinocytes appear to indicate that thapsigargin (but not BAPTA-AM) reduces HD induced DNA fragmentation, but not other endpoints that are also indicative of apoptosis or toxicity. These intriquing findings are very preliminary and are currently being pursued.

The Role of Protease Activation in HD Induced Cell Death

Summary

Our first goal in this research was to define and quantitate the type of cell death caused by HD. In addition, we wanted to examine certain mechanisms which we believed were important in the initiation of cell death. Some of these mechanisms and their relationship to one another and to various initiators of toxicity are outlined in Fig. 28 of the summary. Finally, we intended to interfere with these mechanisms by using inhibitors of those biochemical events that might initiate apoptosis, such as protease activation. In order to assist us in these studies we compared HD induced apoptosis with that caused by well characterized inducers of apoptotic cell death and asked ourselves "Does HD produce apoptosis in a similar manner to other initiators of apoptosis?". Dexamethasone is a compound which has been proposed to upregulate ATP receptors, particularly of the $P2X_1$ subtype, cause calcium influx and activate proteasome, as well as caspase activity. We have now completed studies where we have investigated the effect of the proteasome inhibitors, MG-132 and lactacystin, as well as the caspase inhibitors ZVAD-fmk, IFTD-CHO AND LEHD-CHO. Although these compounds have previously been reported to inhibit cell death resulting from X-irradiation or dexamethasone, they were ineffective in reducing HD induced toxicity, leading us to the conclusion that HD induced apoptotic cell death is unique from that caused by X-irradiation or dexamethasone.

Results

The viability of CHO-K1 cells following HD exposure was assessed using alamarBlue. HD induced a concentration dependent decline in viability that was unaffected by pretreatment with 1-30 μM MG-132 or 3-50 μM lactacystin (Fig. 14). Similarly, HD induced DNA fragmentation was also unaffected by these proteosome inhibitors (Fig. 15). Figure 16 shows that HD caused caspase-3 activation and that this activation was not affected by either MG-132 or lactacystin.

At a concentration of 400 μM, HD induced a clear activation of caspase-3 activity in CHO-K1 cells which was inhibited by the general caspase inhibitor ZVAD-fmk (Fig. 17). Nevertheless, this caspase inhibitor, as well as the more specific caspase inhibitors IFTD-CHO and LEHD-CHO, were ineffective in reducing HD induced toxicity (Fig. 18).

Discussion

The role of proteases has long been a subject of investigation to researchers interested in the actiology of HD induced cell death. Proteasomes are multi-catalytic high molecular mass protease complexes which, upon alteration of their activity, may induce or inhibit apoptosis (Shinohara *et al.*, 1996; Hirsch *et al.*, 1998). Inhibitors of proteasomes have been shown to

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inhibit apoptosis induced by glucocorticords and several other factors (Hirsch *et al.*, 1998). As opposed to the limited research activity devoted towards the role played by proteasomes in apoptosis, a great deal of interest has been directed towards the control of cell death by caspases. These proteases have now been widely implicated as mediators of the initiation of apoptosis, perhaps following activation by elevated intracellular Ca^{2+} levels. Proteases have become excellent candidates to subserve functions relating to apoptosis. Since the toxicity of a variety of apoptotic inducers can be reduced by caspase inhibitors, we examined the effects of these drugs on the viability of CHO-K1 cells following HD exposure.

The proteasome inhibitors, lactacystin and MG-132 were ineffective in modulating HD induced toxicity, as were the specific caspase inhibitors. Caspase-8 and caspase-9 are procaspases and are upstream of caspase-3. Therefore the lack of activity for the specific caspase-8 inhibitor (IFTD-CHO) and caspase-9 inhibitor (LEHD-CHO) can be explained by assuming that more than one pathway leads to caspase-3 activation or that HD directly activates caspase-3. Indeed, as previously reported (Rosenthal *et al.*, 1998), HD was shown to activate caspase-3, results that suggested that this protease played a significant role in the development of HD induced toxicity. However, even though ZVAD-fmk inhibited this activity, it failed to confer any protection against HD toxicity. The lack of protective activity of the general cytosolic caspase inhibitor ZVAD-fmk, which affects caspases 1,3, 8, 7, and 9, seems to be inconsistent with its published characteristics. Previous reports suggest that apoptosis caused by a variety of stimulae can be attenuated by inhibitors of caspases (Bruno *et al.*, 1992; Fearnhead *et al.*, 1995; Weaver *et al.*, 1993). Once again, these results strongly suggest that HD induced initiation of apoptosis is not similar to apoptosis induced by more commonly studied stimulae.

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The Role of ATP- P2X Receptors in HD Induced Cell Death

Summary

As outlined in last year's report and in different areas in this one, one of our goals was to examine the possible roles of P2X receptor subtypes in the initiation of cell death. The $P2X_1$ subtype of receptor has sequence homology with the product of the RP-2 gene, a gene which encodes for apoptosis. On the other hand, the $P2X_7$ subtype of receptor exists on the membrane and acts under lower intensity stimulations by ATP as a calcium pore allowing ATP (or drug induced) Ca^{2+} influx. In addition to the Ca^{2+} pore opening upon more robust stimulation of the receptor, either as a result of higher agonist concentrations or longer exposures, a second pore then opens allowing passage into the cell of larger molecules up to 700 daltons (Hickman *et al.*, 1996; Wiley *et al.*, 1996). Opening of this pore and movement of substances through it can be followed visually by measuring the influx of fluorescent dyes, such as YOPRO-1, which binds to DNA and produces a flourescent signature. The opening of the $P2X_7$ molecular pore often leads to cell death by either apoptosis or necrosis. Since HD causes both types of cell death, the role of principally the $P2X_7$ receptor was examined. We identified the presence of $P2X_7$ receptors in CHO-K1 cells, a finding that the literature indicates would make ATP and BzATP cytotoxic to these cells and whose cytotoxicity should be blocked using specific $P2X_7$ inhibitors, for example oxidized ATP. Nevertheless, we could not demonstrate any toxicity using either ATP or BzATP. Interestingly, however, oxidized ATP was shown to dramatically reduce HD induced DNA fragmentation. However, when HD induced morphology was assessed, it was clear that cell death had been shifted from a primarily apoptotic one, to one predominantly necrotic in nature and that the overall viability status of the cells remained largely unchanged.

Results

The results in Fig. 19 reveal a robust expression of mRNA for the $P2X_7$ receptor in CHO-K1 cells following amplification by RT-PCR. A fragment consistent with the $P2X_1$ receptor is also seen, but was very faint, revealing the predominance of the $P2X_7$ subtype.

The effects of oxidized ATP, a selective inhibitor of the $P2X_7$ receptor (Murgia *et al.*, 1993), on the expression of HD toxicity in CHO-K1 cells were next investigated. HD was incubated with CHOK-1 cells for 5 h, either alone, or following pre-exposure of the cells for 2 h with oxidized ATP. Figure 20 shows the results of agarose gel electrophoresis of DNA extracted from CHOK-1 cells treated with HD alone or in the presence of oxidized ATP (500 μM). HD (500 μM + 600 μM, lane 3 & 4) caused internucleosomal DNA fragmentation and produced a DNA ladder characteristic of apoptotic cell death. Oxidized ATP (Lane 6 & 7) markedly reduced this laddering effect. Quantitative comparisons of HD induced DNA fragmentation in CHO-K1 cells showed a similar trend (Fig. 21), where a 2 h preincubation of the cells with oxidized ATP almost completely eliminated the concentration dependent increase in soluble DNA induced by HD. Additional experiments were carried out using acridine

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orange/ethidium bromide to determine the effects of oxidized ATP on the morphological changes induced by HD in CHO-K1 cells. HD induced morphology characteristic of cells undergoing apoptotic cell death, with shrunken cell bodies and condensed and fragmented nuclei (Fig. 22). Once again, preincubation with oxidized ATP largely eliminated the percentage of cells undergoing apoptosis. However, this assay showed that this treatment produced a concomitant increase in the percentage of cells undergoing necrotic cell death.

The results in Fig. 23 reveal that exposure of CHO-K1 cells to large concentrations of ATP or BzATP, a selective $P2X_7$ agonist, failed to induce a significant cytotoxic response as measured by alamarBlue. Similarly, HD induced cell death was not significantly affected by either of the two ATP analogues (Fig. 24).

Discussion

The results obtained in these studies (Figs. 19-24) are difficult to explain at the present time. We have definitively identified the presence of $P2X_7$ receptors in CHO-K1 cells. Based on this finding, a wealth of scientific literature indicates that 1) both ATP and BzATP should be cytotoxic to these cells, 2) that toxicity is mediated through the $P2X_7$ receptor and 3) that this toxicity can be interrupted by using $P2X_7$ receptor inhibitors such as oxidized ATP. Although CHO-K1 cells were shown to be populated with $P2X_7$ receptors, we could not demonstrate any cytotoxic effects of either ATP or BzATP, in CHO-K1 cells or in any of the other cell types that we have tested.

This apparent disconnect between our data and the literature is of critical importance. Much of the literature infers ATP induced toxicity by using dye uptake studies, where the opening of a pore of sufficient size to allow the uptake of fluorescent markers, is taken as indicative that an osmotic imbalance will occur with subsequent cell death. The toxicity is usually determined using flow cytometric studies, LDH assays where very small LDH increases are taken as toxicity, and visual assessment. As described elsewhere in this report, we are now in the process of repeating these toxicity studies in a different buffer system which should make the ATP receptors, particularly the $P2X_7$ subtype, more accessible to the agonists (Michel *et al.*, 1998; Virginio *et al.*, 1997). It is also hoped that we can examine these cells by flow cytometry in an attempt to demonstrate ATP induced apoptosis by what might prove to be a more sensitive measure of this parameter. In addition, the functional aspects of the ATP receptor in CHO-K1 cells are currently being studied to examine the effect of BzATP and HD on the uptake of the fluorescent dye YOPRO-1, which would confirm the presence and functional activity of the $P2X_7$ receptor (Steinberg *et al.*, 1987).

Although we were not successful in detecting ATP or BzATP toxicity in CHO-K1 cells, we showed that oxidized ATP, a specific $P2X_7$ inhibitor, was extremely effective in reducing the HD induced DNA ladder patterns that are characteristic of apoptosis. This drug also reduced soluble DNA in these cells. Interestingly, however, when the overall viability of the cells was assayed using alamarBlue (data not shown), no protection was observed. Further studies investigated the effects of oxidized ATP on HD induced morphology and showed that although the apoptotic aspects of the morphological changes induced by HD were reduced, the percentage of necrotic cells was dramatically increased. Clearly, the action of oxidized ATP was to shift the mechanism of cell death to one that was predominantly necrotic in nature. We are currently pursuing these studies.

Further Identification of P2X Receptors in Synaptosomes

Summary

In last year's summary we reported the identification of $P2X_7$ ATP receptors on neuronal synaptosomal terminals and that HD, unlike ATP, failed to induce calcium influx. It has been recently reported that buffers with high ionic content (similar to the buffers that we use), inhibit the activity of P2X receptors. Therefore, we also carried out additional studies where we substituted the sodium in sodium chloride based buffers and media with either choline or sucrose. These two buffers have been reported to apparently alter the affinity of the ATP analogues to the P2X receptors, particularly the $P2X_7$ receptor (Michel *et al.*, 1998; 1999; Virginio *et al.*, 1997). We report here studies carried out with these new buffer/medium formulations in the investigations of ATP induced calcium influx and intracellular free calcium levels (nerve endings) in synaptosomes and show the importance of the ionic environment when examining P2 receptor function.

Results

The results in Fig. 25 reveal that BzATP induced Ca^{2+} influx in synaptosomal preparations was markedly potentiated when the sodium chloride buffer was substituted by choline, and was even further enhanced when sucrose was substituted. Figure 26 reveals a similar pattern in the measurement of intracellular free calcium levels. The response was barely measurable in synaptosomes exposed to ATP in sodium chloride based buffer. However, when this was replaced by sucrose, BzATP caused a rapid and robust response. The effects of Brilliant Blue G (BBG), a selective $P2X_7$ inhibitor, are shown in Fig. 27. In normal sodium chloride buffer BBG was ineffective in inhibiting BzATP evoked Ca^{2+} influx in synaptosomes (data not shown), but was quite potent in decreasing influx when the assays were carried out in sucrose buffer.

Discussion

The results obtained with the synaptosomal preparations are entirely consistent with published results concerning the inhibitory activity of certain ions on the activity of P2X receptors. These previous studies have shown that ATP receptors, especially of the $P2X_7$ subtype, are tonicly inhibited in certain buffers. Buffers which contain sodium chloride appear to inhibit receptors more than those containing choline chloride, while the least amount of inhibition is observed in the cation free sucrose buffers. In addition, we have found that selective ATP inhibitors are more potent in buffers with reduced ionic composition. All of these results are consistent with findings published in last year's report, where $P2X_7$ receptors were identified by several techniques and that they control calcium influx and to some degree, neurotransmitter release from presynaptic nerve endings (a finding of considerable importance in itself). We are currently examining the interaction of HD and BzATP on this system in

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sucrose buffer. These new results also open new avenues for the study of ATP induced apoptosis in other cell types, which to date have been resistant to ATP induced cell death. The high cationic concentrations in the buffers/media that we have previously used may have been responsible for our inability to demonstrate the ATP induced apoptosis which is widely reported in the literature. It is our plan to repeat some of our ATP cytotoxicity studies in media in which the ionic composition has been lowered (Michel *et al.*, 1999), or in which the concentration of certain divalent cations has been reduced (Virginio *et al.*, 1997). This topic is further discussed in the Discussion/Summary below.

Problems Encountered and Solutions Proposed In Demonstrating ATP induced Cell Death

The original proposal for this co-operative agreement was based on several inter-related observations found either in the literature or in our own laboratory. They consisted of what we will call (a) established "fact", (b) observations from our own laboratory and (c) hypothesis which utilized the facts and observations to suggest a plausible testable theory. The fact was that ATP was a potent cytotoxic agent when applied to the membranes of a variety of cells. The receptors on these cells were capable of initiating cell death following ATP receptor stimulation, which was believed to initiate Ca^{2+} influx and, depending on the ATP receptor, the entrance of molecules up to 700 daltons. These two events led to the proposal that HD induced cell death was the result of increased intracellular Ca^{2+} levels and some form of osmotic disruption directly linked to ATP receptor activity.

Several theories have been put forth suggesting that HD might produce cell death by activating some type of biochemical trigger that was in turn responsible for major changes in cellular architecture. It has been theorized that HD induced cell death might be the result of either apoptosis, necrosis, or both mechanisms and further, that these events may be the result of an elevation of intracellular Ca^{2+} (Papirmeister *et al.*, 1991). Our original hypothesis, based on experimental evidence (Lundy *et al.*, 1998; Hamilton *et al.*, 1998) included the suggestion that HD and ATP were quite capable of interacting with one another, at least at certain ATP receptor subtypes. It was proposed then, that they might be synergistic and that HD and other apoptotic inducers might conformationally change the structure of ATP receptors to make ATP a more efficient effector of apoptosis.

Most of the data that we have gathered to date are not inconsistent with our initial proposal. In fact, the more recent evidence, particularly with CHO-K1 cells, is quite exciting in this respect. However, we are having great difficulty reproducing the "fact" portion of the proposal. Although a large number of papers have appeared illustrating ATP-induced apoptosis following $P2X_1$ and $P2X_7$ receptor stimulation and upregulation, we at this point have not been able to reproduce these studies. We originally believed that the demonstration of ATP-induced cell death would constitute the easiest part of the proposal to complete. Therefore, we have spent considerable efforts in manipulating cultures in an attempt to demonstrate the induction of apoptosis following ATP or its analogues. We have not included this data here, other than to report that we have been surprisingly unsuccessful in detecting measurable ATP induced toxicity of any type, in a variety of different cell types.

There are two major approaches used to assess the occurrence of apoptosis in different cell types. One approach relies on biochemical markers such as the production of DNA fragments (ladders), annexin V staining, TUNEL reaction, etc., and the second is the use of flow cytometry. The majority of those studies in which ATP-induced apoptosis has been demonstrated, have used flow cytometry as an end point to define apoptosis. We, on the other hand, have used biochemical markers. We suggest, therefore, that we carry out flow cytometric studies to see if there are major differences in the outcome with respect to the definition of apoptosis following ATP. Obviously, we have had no difficulty at all in the determination of apoptosis following cellular exposure to HD in our studies.

The second remedy is one that we have just begun and also looks promising. Several recent reports now suggest that ATP induced receptor activation is under tonic inhibition by certain ions (Michel *et al.*, 1998; 1999). Therefore, experiments are underway in which non-ionic buffers (described in the references above) have been substituted for traditional ionic buffers. Initial experiments have yielded positive results with respect to the observation that P2X agonists have now produced DNA ladders in CHO-K1 cells. We our now repeating some previous experiments in this new buffer.

As a consequence of how the work on this proposal has progressed, we would like to request an additional year to finish these studies. We need no further resources to carry this out, since we now have access to both flow cytometry instrumentation and expertise. We have attached a formal letter outlining this request.

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Key Research Accomplishments

1. Expansion and completion of last year's results which demonstrate that HD induced primarily apoptosis, and to a lesser degree necrosis, in a diverse number of cell types

2. Despite the important observation that HD induced increases in intracellular calcium levels in several different cell types, modulation of the external and internal calcium levels failed to offer proof of any causality between toxic effects and calcium levels. The discovery that low ionic strength buffers enhanced the calcium mediated responses to ATP and its analogues was important in relating ATP receptor activation to cell death (see below).

3. HD caused induction of caspase-3, a critical determinant of apoptosis. However, caspase inhibitors failed to inhibit HD-induced cell death.

4. Although the apoptosis induced by several different apoptotic stimulae is antagonized by inhibitors of proteasomes, HD induced apoptosis was unaffected by proteasome inhibitors.

5. P2X receptor subtypes were identified with some certainty in neuronal synaptosomes, CHO-K1 and J774 cells.

6. New low ionic strength buffers were adopted which facilitated the observation that ATP induced apoptosis in CHOK-1 cells was mediated through a $P2X_7$ receptor subtype. (This was a major accomplishment due to our previous inability to demonstrate ATP induced cytotoxicity in commonly used media and/or buffers. We also observed that HD-induced apoptosis in these cells was inhibited by oxidized ATP, a specific inhibitor of $P2X_7$ receptors. This is evidence of a link between ATP receptor activity and some aspects of HD-induced toxicity.

7. Preliminary evidence was obtained in both keratinocytes and CHO-K1 cells that show that inhibition of the $P2X_7$ mediated HD-induced apoptosis by oATP does not reduce cytoxicity because the cells shunt to a necrotic pathway. These results are being expanded.

8. Several lines of evidence point towards HD being a unique inducer of apoptosis, in that several types of inhibitors that are effective in preventing apoptosis induced by other more characterized apoptotic stimulae, for example dexamethasone and X-irradiation.

Reportable Outcomes

1. Lundy, P.M., Gong, W., Mi, L., Nelson, P., Vair, C., Frew, R., Sawyer, T.W. and Hamilton, M.G. (1999). Mediation of sulfur mustard cellular toxicity by ATP: A possible mechanism of action of sulfur mustard toxicity. *DRES Technical Report* **No. 191**, 49 pages.

2. Gong, W., Mi, L., Nelson, P., Hamilton, M.G., Lundy, P.M. and Sawyer, T.W. (2000). Sulphur mustard induces apoptotic cell death in diverse cell types. *Toxicol. Appl. Pharmacol.* (Submitted).

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Conclusions and Summary

The initiation of apoptosis by chemical stimulae has been the subject of countless papers and review articles and Figure 28 summarizes many of the mechanisms which are thought to play roles in this biochemical process. We have included this diagram to illustrate the hypothesis with which we are working; that ATP and HD may act as a stimulus at a common biochemical locus to initiate apoptotic events. In this report we have characterized HD induced apoptotic cell death in different cultured cell types and documented our attempts to link the cytotoxicity of HD and ATP by investigating the effects of modulating intracellular calcium, protease activation and $P2X_7$ receptor activation.

We have shown that HD exerts its toxicity in a highly conserved fashion in the different cell types that we examined. This observation strengthened the likelihood that similar mechanisms of cell death were also operative *in vivo* and that strategies based on interrupting these well defined events would be successful in protecting against HD induced toxicity. One research direction that we pursued was intracellular calcium, whose elevation has been consistently associated with the triggering of apoptosis. The literature contains numerous references documenting this link between the two processes (Fawthrop *et al.*, 1991; Nicotera and Orrenius, 1998; Kass and Orrenius, 1999) and indeed, HD toxicity has also been suggested as being related to changes in calcium metabolism (see Papirmeister *et al.*, 1991). Although it appears that HD does cause a rise in intracellular calcium (Hua *et al.*, 1993; Mol, 1994; Mol and Smith, 1996; Ray *et al.*, 1994), it is by no means clear at this point what role, if any, that this rise in intracellular calcium plays in the subsequent HD induced toxicity. It should be pointed out that a rise in intracellular Ca^{2+} is not always a prerequisite to the induction of apoptosis (Kass and Orrenius, 1999).

We initially suggested that ATP receptors might provide a convenient site for the calcium entry that we observed in our early smooth muscle studies. We have now examined the source of the Ca^{2+} increases and begun to examine the role and type of P2X receptors in the target cells that might have been responsible for the calcium elevation. The elevation of cellular calcium indeed appears to be an early event following exposure of cells to HD and certain ATP analogues. An examination of the possibility that the increased Ca^{2+} levels observed could trigger secondary activation of internal signals that help determine the fate of the cell has also been initiated with some interesting results. These internal signals, such as caspase activation, the initiation of gene expression and more recently the activation of endonucleases are all known to be extremely important in regulating cell death. An outline of the possible interactions of the various events which we are studying is highlighted in Fig. 28.

Many of our studies have unfolded in a logical fashion while others have not. For example, we have shown HD to be a potent activator of apoptotic events in a number of cell types and also that it produces at least a moderate increase in intracellular Ca^{2+} concentrations in these same cells, apparently from intracellular calcium sources. However, we have not been successful in demonstrating a relationship between these increases and HD induced toxicity. This is critical, because it indicates that HD induced toxicity and calcium elevation may be causally unrelated. In contrast to these findings, very recent experiments in human keratinocytes appear to indicate that thapsigargin (but not BAPTA-AM) may reduce HD

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induced DNA fragmentation, but not other endpoints that are also indicative of apoptosis or toxicity. The significance of these preliminary findings remains to be examined.

The activation of caspase activity (Fig. 28) appears to be a common factor in the production of apoptosis by a variety of stimulae (Cohen, 1997; Hengartner, 2000; Nicholson and Thornberry, 1997; Kidd, 1998) and HD did indeed induce caspase-3 activity (see Fig.17). Therefore, at face value the present data appear to support the hypothesis that HD might exert its toxic effects through caspase activation. (Cohen *et al.*, 1997; Hengartner, 2000; Nicholson *et al.*, 1997; Kidd, 1998). However, as our calcium data revealed, modulating a biochemical parameter does not necessarily alter HD induced toxicity; despite activating caspase-3, HD toxicity was apparently unaffected by caspase inhibition.

Figure 29 illustrates the relationship of dexamethasone and X-irradiation to some of the parameters that we have studied using HD as the initial apoptotic stimulus. It summarizes some of the published results showing that the proteasome inhibitors MG-132 and lactacystin, and several caspase inhibitors such as ZVAD-fmk were effective inhibitors of apoptosis induced by these stimuli (Bruno *et al.*, 1992; Fearnhead *et al.*, 1995; Weaver *et al.*, 1993; Squier *et al.*, 1994). In our studies we were not successful in showing similar responses when the apoptotic stimulus was HD. The emerging pattern of these studies clearly indicates that HD toxicity is largely the result of apoptosis and to a lesser extent necrosis. However, although HD induced apoptosis shares some common characteristics with a number of other apoptotic stimulae, ie. with respect to protease activity and calcium influx, it clearly differs from other initiators with respect to the reversibility of the apoptotic process. HD induced apoptosis was not affected by blocking its effects on calcium or on proteasome activation, nor by blocking its effects on caspase activation (Fig. 28). These differences in reversibility between HD and other inducers of the process, may be the result of the extreme chemical reactivity of HD as compared to other common initiators of apoptosis such as dexamethasone. It is possible, indeed probable, that HD causes cell death through the contribution of many biochemical "lesions" in the affected cell. It is possible that a number of biochemical "sites" may become activated simultaneously and that inhibition of any single parameter would be insufficient to reverse the apoptotic process. It is also possible that inhibition of any one of the HD induced lesions normally leading to apoptosis might intensify whatever changes were necessary to shift the cell into becoming necrotic. This scenario was, in fact, supported with our studies using oxidized ATP, a specific inhibitor of the $P2X_7$ receptor (see below).

We examined the possible role for ATP receptors in HD-induced cell death for several reasons. These included the facts that 1) the pharmacological potentiation of HD and ATP have been reported (Lundy *et al.*, 1998; Hamilton, *et al.*, 1998), 2) ATP causes cell death in a wide variety of cell types (Dubyak and El-Moatassim, 1993) and 3) ATP causes apoptosis through activation of ionic channels which allow both the influx of divalent cations (Ca^{2+}) and the opening of larger pores leading to osmotic lysis, perhaps apoptosis, and likely necrosis. Our early studies devoted towards the identification of ATP receptors in various tissues were successful and we identified the presence of $P2X_7$ receptors in CHO-K1 cells. This suggested that ATP and related analogues would be toxic in this cell type and that specific inhibitors of this receptor would prevent ATP induced cell death and perhaps, HD induced toxicity. Once again, however, our work met with mixed success that was difficult to explain. Although, we could demonstrate no ATP toxicity, oxidized ATP was protective in some respects against HD. Very recent work has demonstrated that in all probability, the presence of high cation

concentrations in the buffer/media has been responsible for our marked lack of success in demonstrating ATP induced effects. Although these special buffers and media seem to us as being rather "non-physiological", we are currently pursuing these studies. The results with the oxidized ATP illustrate our previous assertion that modulating only certain aspects of the cascade of events that leads to cell death may, at times, serve only to shunt the death pathway from one that is predominantly apoptotic to one that is more necrotic in nature. Although this $P2X_7$ inhibitor dramatically reduced the DNA fragmentation induced by HD, necrosis was significantly increased and the overall toxicity of HD treatment in terms of metabolic viability was unchanged.

In summary, this report describes our efforts to both characterize HD induced cell death, as well as our attempts to interrupt this toxicity with specific inhibitors of the defined pathways that we have identified. HD appears to exert its toxicity in a remarkably conserved fashion in different cell types that makes it probable that a strategy based on interfering with the well defined apoptotic events should be successful. However, preliminary findings indicate that HD exerts apoptotic cell death in a fashion that is unique from other, better characterized apoptotic stimulae.

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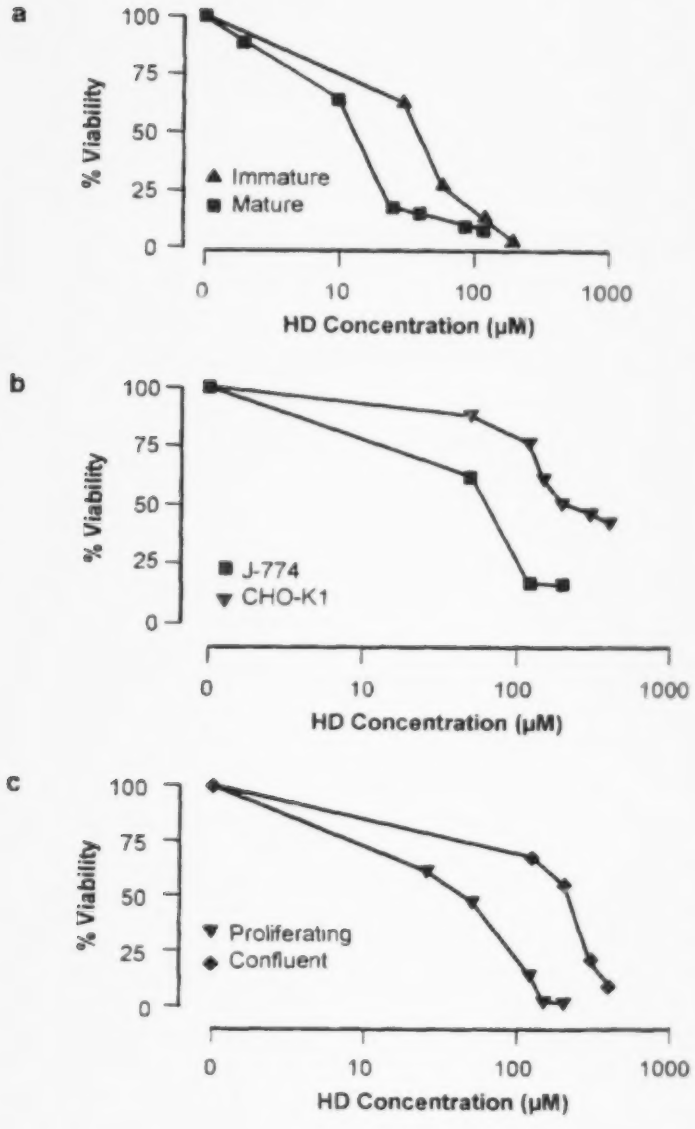

Figure 1. *Toxicity of HD in various cell types Cultures of neurons (a), J774 cells (b), CHO-K1 cells (c) and keratinocytes (d) were treated with HD and cytotoxicity was assessed 24 h(neurons, CHO-K1, J774) or 48 h (keratinocytes) later using alamarBlue Results represent a typical experiment utilizing the means of six wells per data point*

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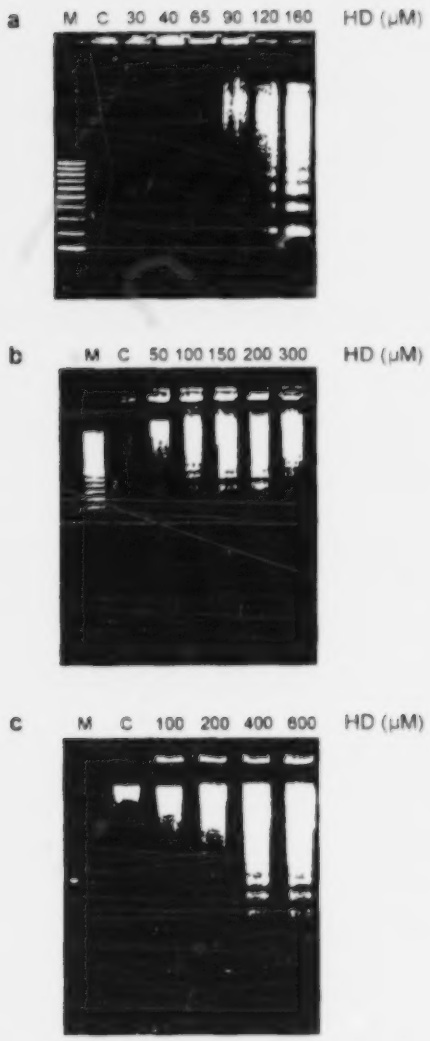

Figure 2. *HD induced DNA fragmentation in various cell types. Cell cultures were treated with varying concentrations of HD, the cells were harvested and then the DNA fragments were extracted and separated on 1.5 % agarose gels. Control cultures showed no DNA fragmentation. HD treatment induced the DNA ladders characteristic of apoptotic cell death in neurons (a), J774 cells (b) and CHO-K1 cells (c).*

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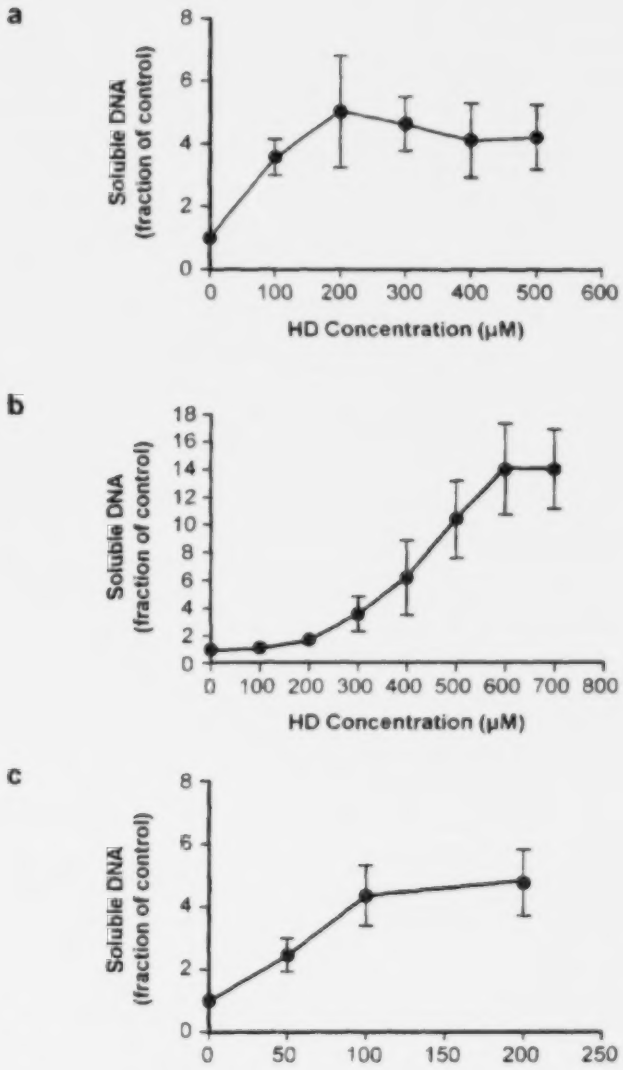

Figure 3. *HD induced soluble DNA in various cell types. Cellular DNA was labeled with tritiated thymidine prior to HD treatment and subsequent quantitation of DNA fragmentation. Results are the mean - - standard deviation of three separate experiments and are normalized against controls. In human keratinocytes (a), CHO-K1 cells (b) and J774 cells (c) HD induced a concentration dependent increase in DNA fragmentation that plateaued at higher HD concentrations.*

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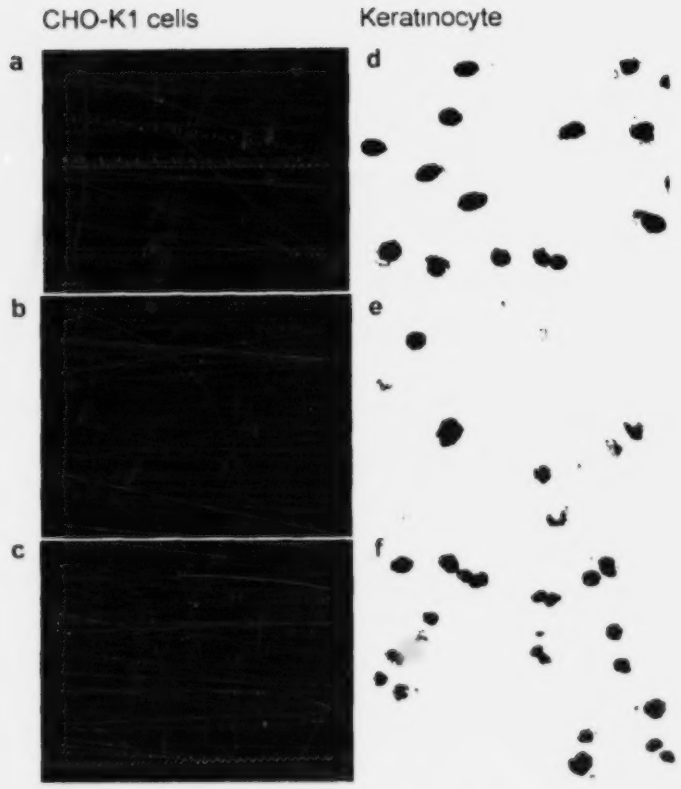

Figure 4. *Detection of HD induced apoptotic cell death in cell culture by the TUNEL reaction. CHO-K1 and human skin keratinocytes were treated with HD and then assayed for apoptotic cell death using the TUNEL reaction and fluorescent microscopy (4a-c) or light microscopy (d-f), respectively. No apoptotic cells in vehicle treated control cultures (a, d) were detected. Concentration-dependent increases in fluorescein labeled nuclei were observed in CHO-K1 cells treated with 400 μM HD (b) and 600 μM HD (c) with condensed chromatin clearly visible. Similarly, brown apoptotic nuclei are visible in keratinocytes treated with 200 μM HD (e) and 400 μM HD (f).*

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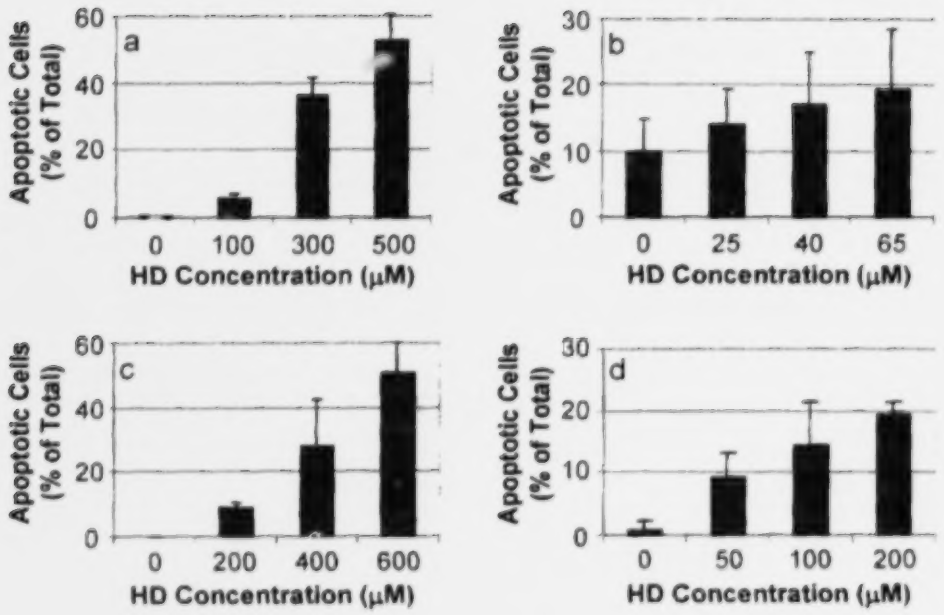

Figure 5. *Quantitation of HD induced apoptotic cell death in various cell types by the TUNEL assay. Cells were treated with HD and then assayed for apoptotic cell death using the TUNEL reaction. With the exception of neuronal cells, very few vehicle treated control cells were apoptotic. The percentage of apoptotic cells increased with HD concentration in human keratinocytes (a), chick neurons (b), CHO-K1 cells (c) and J774 cells (d). Results represent the mean ± standard deviation of at least three separate experiments.*

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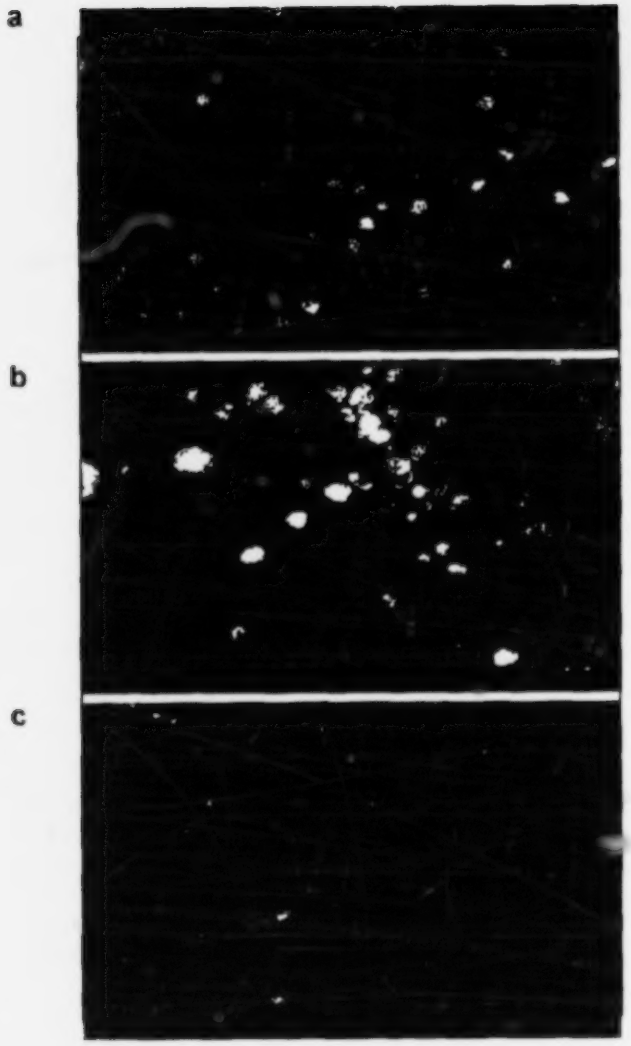

Figure 6. *Detection of HD induced apoptotic cell death using morphological criteria. Cultures were treated with HD and then morphology was assessed using ethidium bromide and acridine orange. Figure 6a-c shows an example of the effects of HD on J774 cells when assayed morphologically. Control cells (a) show normal green nuclei while 50 μM HD induced the appearance of apoptotic nuclei (b, bright yellow green condensed or fragmented chromatin). At higher HD concentrations (200 μM, c) the cell membrane loses its integrity and ethidium bromide enters the cell, staining the chromatin orange.*

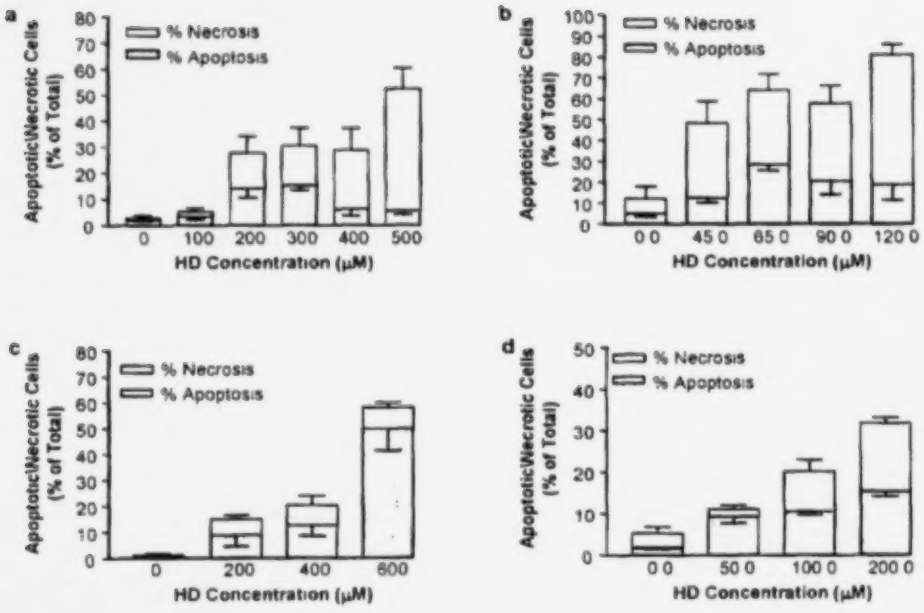

Figure 7. *Quantitation of HD induced cell death using morphological criteria The effects of HD on the morphology of human skin keratinocytes (a), chick neurons (b), CHO-K1 (c) and J774 cells (d) was assessed using acridine orange and ethidium bromide*

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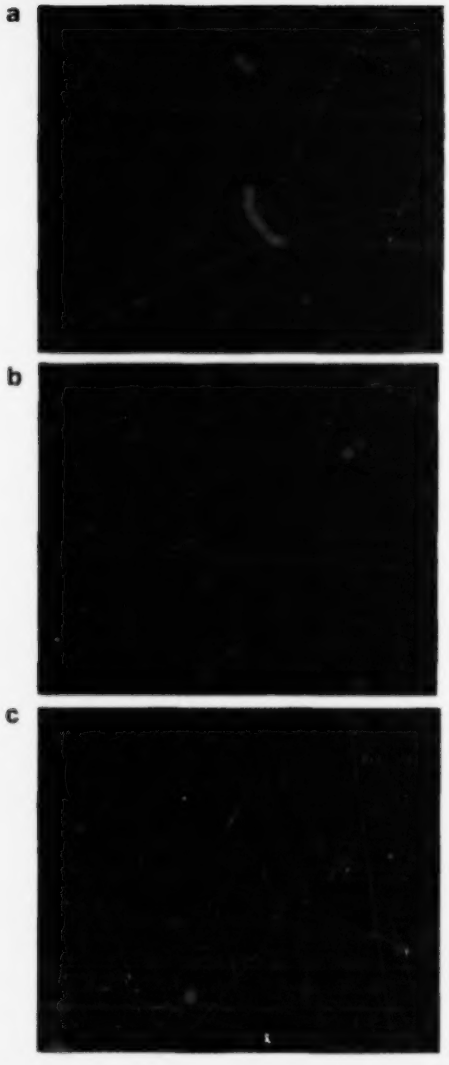

Figure 8. *Detection of HD induced apoptotic cell death using Annexin V in CHO-K1 cells. Control cells (a) are generally negative. At 200 μM HD (b) the fluorescein labeled antibodies recognize the externalized phosphotidylserine residues that signify early stage apoptotic cell death and stain the cells green. At higher concentrations of HD (c, 400 μM), the cell membrane loses integrity and increasing numbers of cells are also stained with propidium iodide (red brown colour) indicating necrosis.*

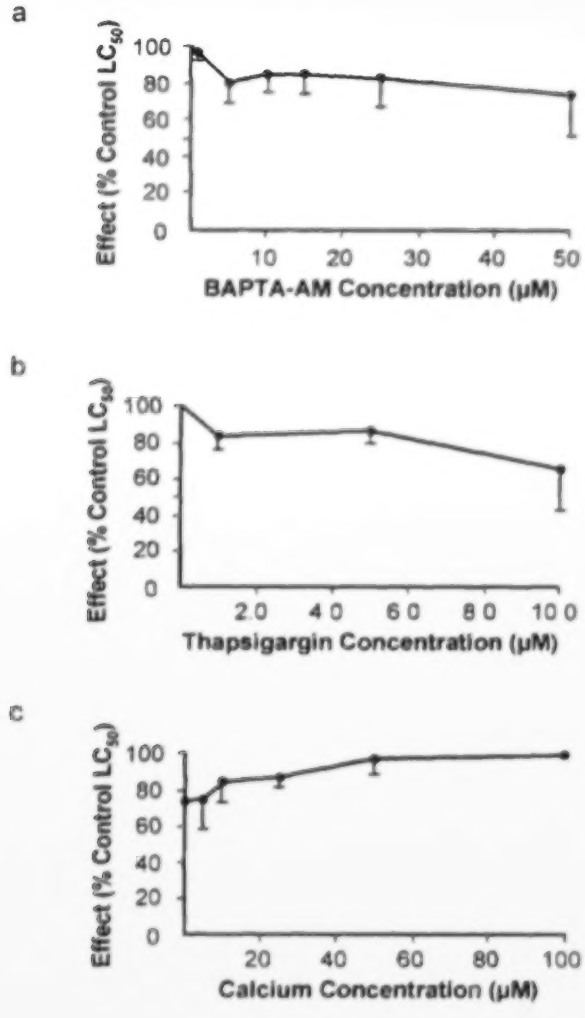

Figure 9. *Effect of intracellular calcium modulation on HD toxicity in keratinocytes. The effect of BAPTA-AM (a), thapsigargin (b) or nominal zero external calcium concentrations (c) were examined in confluent cultures of human skin keratinocytes. One hour prior to HD treatment, the medium bathing the cultures was changed to treatment medium containing 25 μM BAPTA-AM, 10 μM thapsigargin or nominal zero calcium concentrations. The cultures were treated with HD and then cellular viability was assessed 48 h later using alamarBlue. The results are expressed as a percentage of the LC_{50} of sham pretreated, HD treated cultures. No pretreatment had any effect on the toxicity of HD. Results represent the mean ± standard deviation of at least three experiments.*

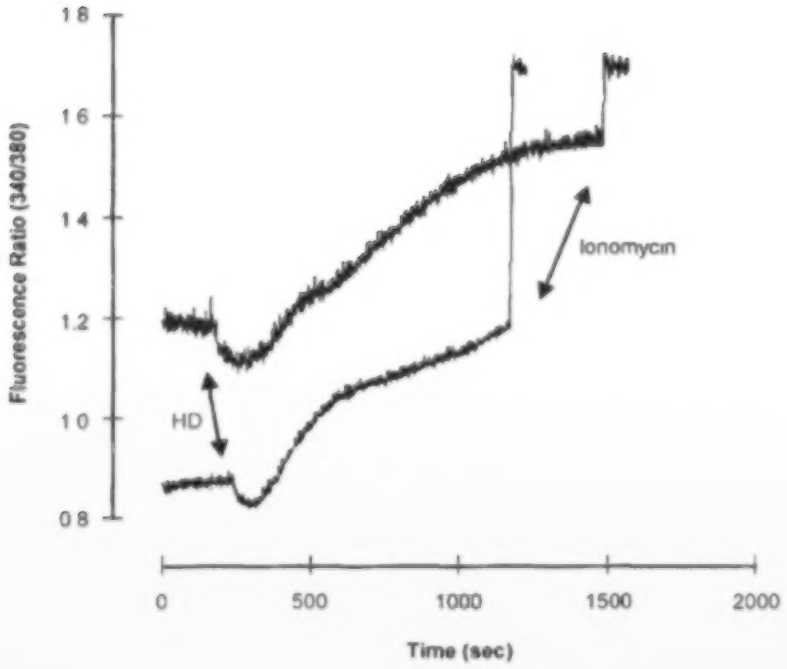

Figure 10. *Effect of HD on intracellular calcium levels in J774 and CHO-K1 cells The addition of 600 μM HD caused a slow increase in the 340 380 nm fluorescence ratio (indicative of intracellular calcium) in both CHO-K1 (top) and J774 (bottom) cells.*

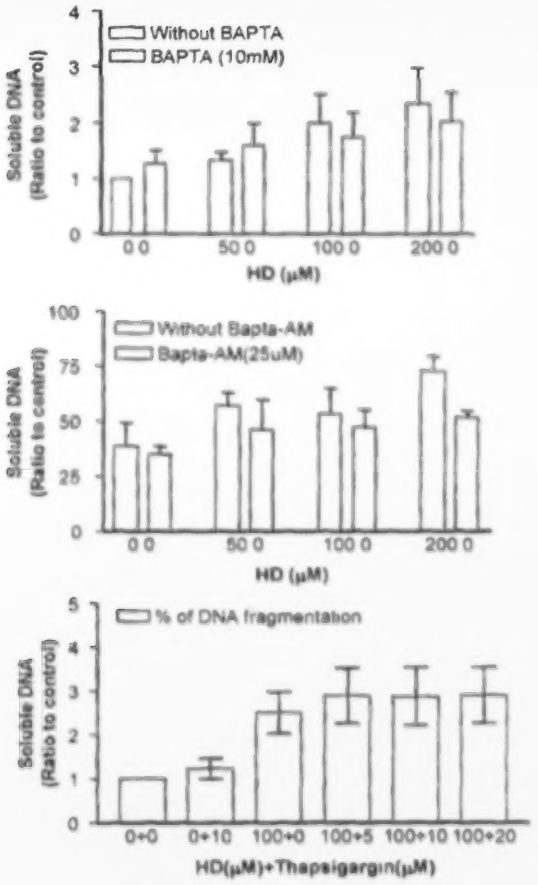

Figure 11. *Effect of intracellular calcium modulation on HD induced soluble DNA in J774 cells Tritiated thymidine labeled cells were incubated with or without BAPTA (a, to chelate external calcium), BAPTA-AM (b) or thapsigargin (c) for 1 h prior to HD exposure Four hours later soluble DNA was isolated No pretreatment had any effect on HD induced soluble DNA Results represent the mean + - standard error of the mean of three experiments*

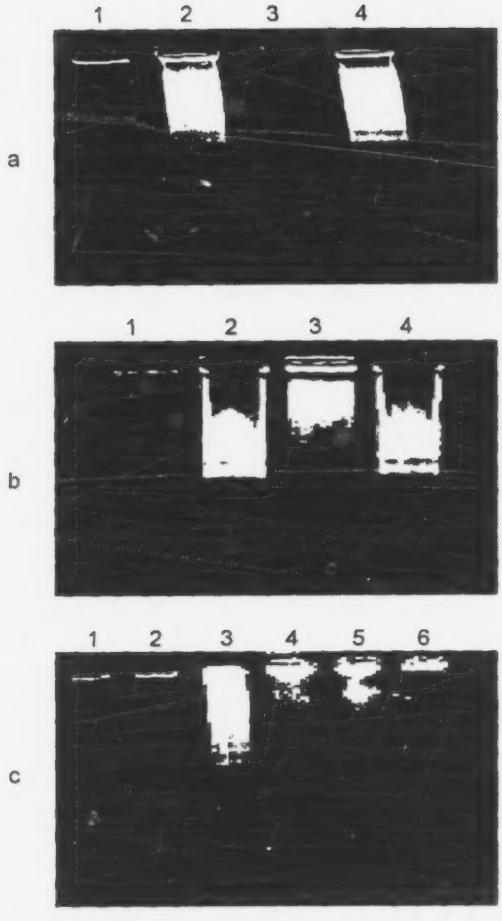

Figure 12 *Effect of intracellular calcium modulation on HD induced DNA fragmentation in J774 cells Panels show electrophoresed gels of DNA extracted from cells treated in a similar manner to that described in Fig 11 No pretreatment altered HD induced ladders of DNA*

Panel (a) lane 1, 0 μM HD/0 mM BAPTA, lane 2, 100 μM HD/0 mM BAPTA, lane 3, 0 μM HD/10 mM BAPTA, lane 4, 100 μM HD/10 mM BAPTA

Panel (b) lane 1, 0 μM HD/0 μM BAPTA-AM, lane 2, 100 μM HD/0 μM BAPTA-AM, lane 3, 0 μM HD/25 μM BAPTA-AM, lane 4, 100 μM HD/25 μM BAPTA-AM

Panel (c) lane1, 0 μM HD/0 μM thapsigargin, lane 2, 0 μM HD/10 μM thapsigargin, lane 3, 100 μM HD/0 μM thapsigargin, lane 4, 100 μM HD/5 μM thapsigargin, lane 5, 100 μM HD/10 μM thapsigargin, lane 6, 100 μM HD/20 μM thapsigargin

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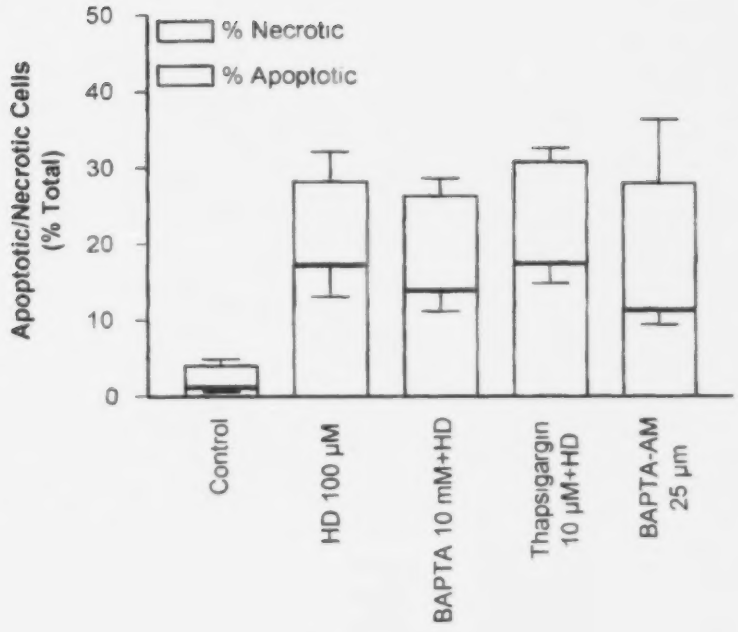

Figure 13. *Effect of intracellular calcium modulation on HD induced morphology in J774 cells Cells were treated in a similar manner to that described in Fig 11 and then HD induced morphology was assessed as described in the Methods section No pretreatment had any significant effect on the mode of cell death Results represent the mean + - standard error of the mean of three experiments*

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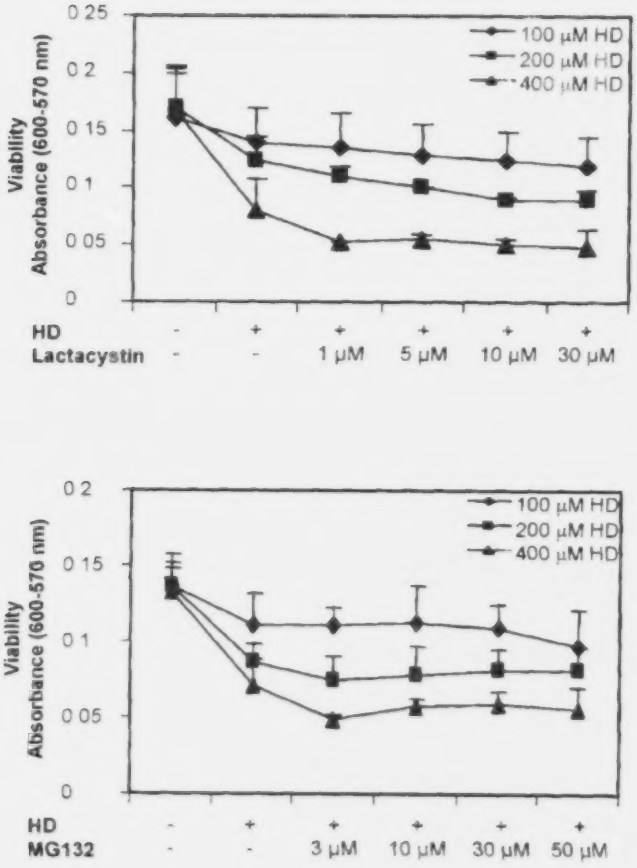

Figure 14. *Effect of proteosome inhibition on HD induced cytotoxicity in CHO-K1 cells CHO-K1 cells were incubated with MG132 or lactacystin for 15 min prior to HD treatment The cells were then allowed to incubate for a further 24 h after HD treatment. The viability of the cells was then assessed using the metabolic indicator dye alamarBlue Neither MG132 nor lactacystin significantly modified HD induced cell death. Results represent the mean - - standard error of the mean of three experiments*

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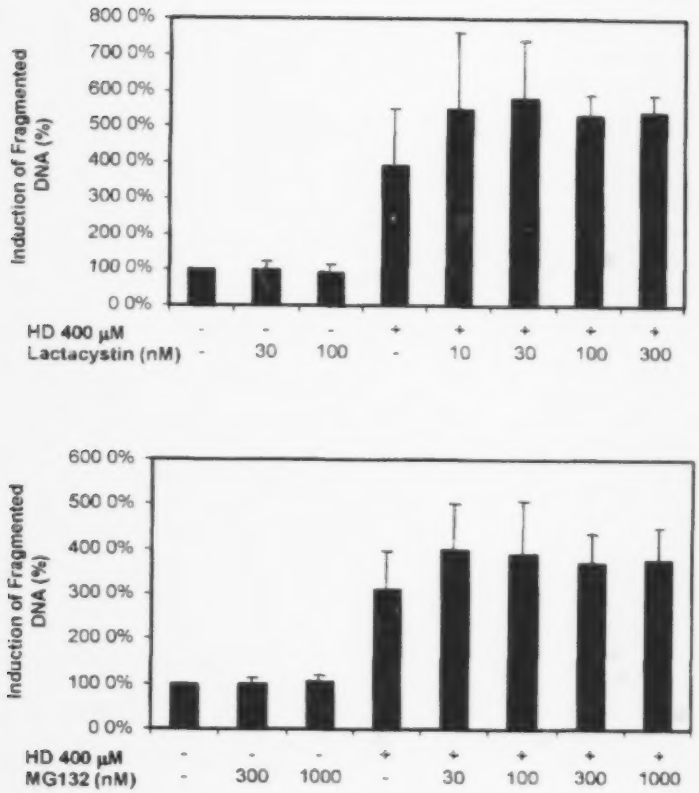

Figure 15. *Effect of proteosome inhibition on HD induced soluble DNA in CHO-K1 cells. CHO-K1 cells were incubated with MG132 or lactacystin for 15 min prior to HD treatment. The cells were then allowed to incubate for a further 6 h after HD treatment. DNA fragmentation was quantitated as described in Methods. Treatment of the cells with 400 µM HD resulted in an increase in soluble DNA of approximately 300-400%. Treatment with the proteasome inhibitors did not significantly alter*

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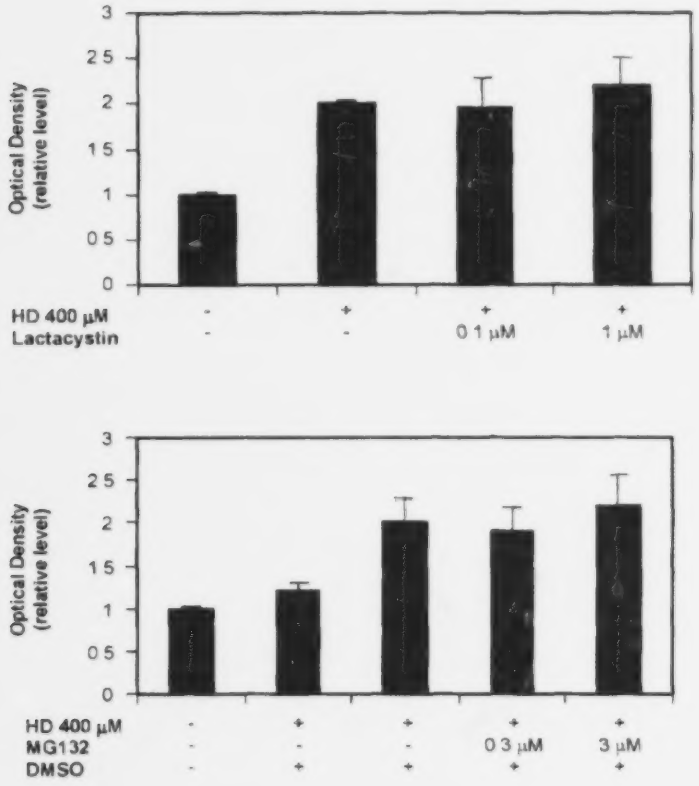

Figure 16. *Effect of proteosome inhibition on HD induced caspase-3 activity in CHO-K1 cells. CHO-K1 cells were incubated with MG132 or lactacystin for 15 min prior to HD treatment. The cells were then allowed to incubate for a further 6 h after HD exposure. Treatment of the cells with 400 µM HD resulted in an elevation of caspase-3 activity. However, treatment with the proteasome inhibitors did not significantly change this HD induced elevation. Results represent the mean + - standard error of the mean of three experiments.*

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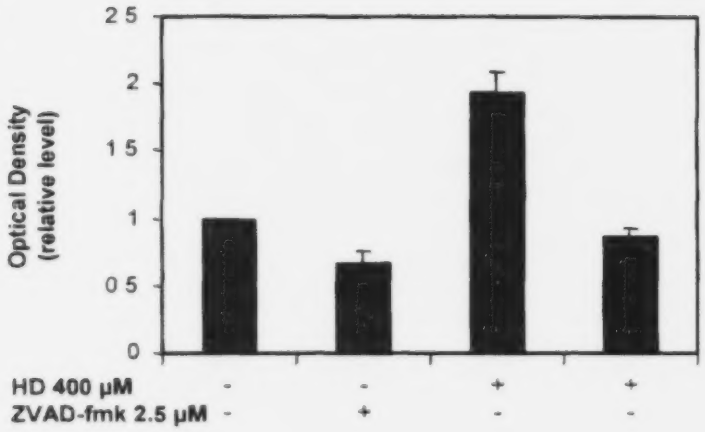

Figure 17. *Effect of caspase inhibition on HD induced caspase-3 activity in CHO-K1 cells CHO-K1 cells were incubated with the general caspase inhibitor ZVAD-fmk for 15 min prior to HD treatment The cells were then treated with HD and allowed to incubate for a further 6 h Exposure of the cells with 400 µM HD resulted in an elevation of caspase-3 activity Pretreatment of both control and HD treated cells with ZVAD-fmk resulted in a significant depression in both constitutive and induced caspase-3 activity Results represent the mean +/- standard error of the mean of three experiments*

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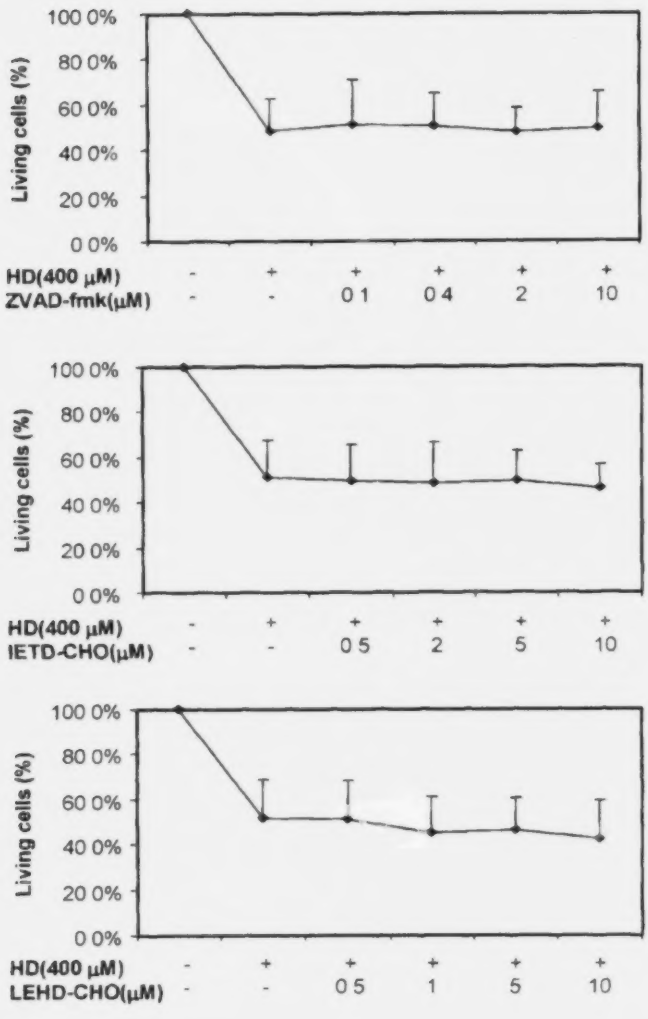

Figure 18. *Effect of caspase inhibitors on HD induced cytotoxicity in CHO-K1 cells. CHO-K1 cells were incubated with either the general caspase inhibitor, ZVAD-fmk, or the specific caspase inhibitors, IEHD-CHO and LEHD-CHO for 15 min prior to HD treatment. The cells were then allowed to incubate for a further 24 h after HD treatment. The viability of the cells was assessed using the metabolic dye indicator alamarBlue. None of the caspase inhibitors altered HD toxicity. Results represent the mean - - standard error of the mean of three experiments.*

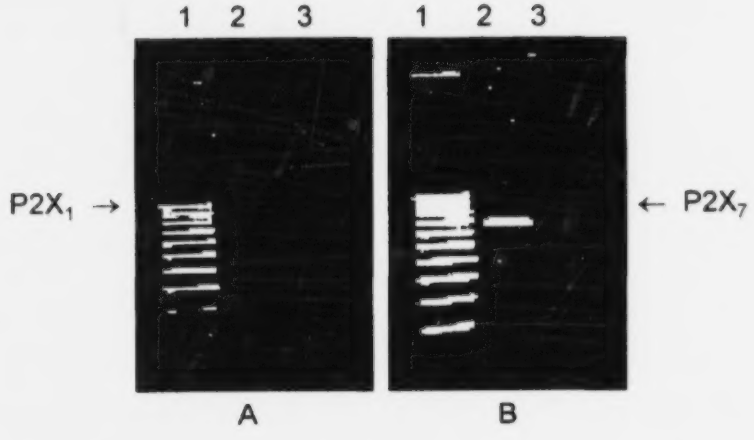

Figure 19. *Expression of $P2X_1$ and $P2X_7$ receptor mRNA from CHO-K1 cells RT-PCR was used to examine the transcript expression of $P2X_1$ and P2X- receptors in CHO-K1 cells Reaction with the total RNA from CHO-K1 cells without reverse transcription was used as the negative control (lane 3, both panels) Lane 1 shows the 100 base pair molecular weight markers, while lane 2 shows the amplified fragment of 594 bp ($P2X_1$, panel A) and 660 bp (P2X-, panel B), respectively*

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Figure 20. *Effect of oxidized ATP on HD induced DNA fragmentation in CHO-K1 cells. Agarose gel of DNA extracted from CHO-K1 cells treated with HD for 5 h, or of cells treated with oxidized ATP for 2 h prior to HD treatment. Lane 1, 100 bp marker, lane 2, 0 μM HD, lane 3, 500 μM HD, lane 4, 600 μM HD, lane 5, 500 μM HD/500 μM oxidized ATP; lane 6, 600 uM HD/500 μM oxidized ATP. Oxidized ATP eliminated the DNA ladders induced by HD*

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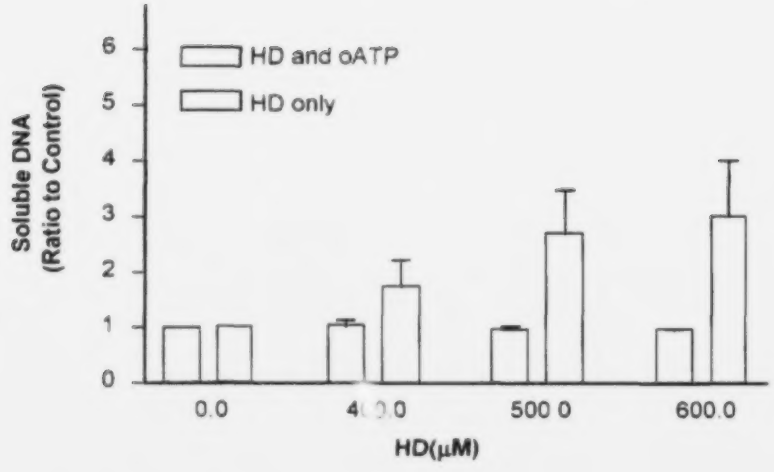

Figure 21. *Effect of oxidized ATP on HD induced soluble DNA in CHO-K1 cells. Tritiated thymidine labeled CHO-K1 cells were incubated with various concentrations of HD for 4 h with or without a 2 h preincubation with 500 μM oxidized ATP. Soluble DNA was then isolated and quantitated. Results represent the mean - - standard error of the mean of three experiments. Oxidized ATP eliminated the soluble DNA induction by HD.*

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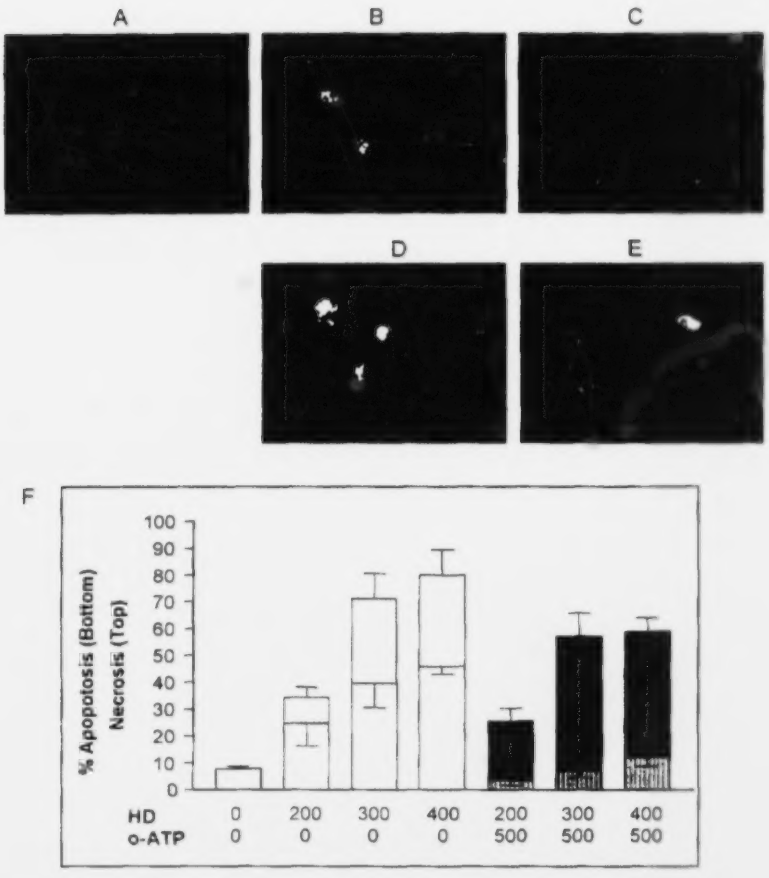

Figure 22. *Effect of oxidized ATP on HD induced morphology in CHO-K1 cells Acridine orange-ethidium bromide was used to stain CHO-K1 cells treated with HD in the presence or absence of oxidized ATP Panel A, control, panel B, 300 μM HD, panel C, 400 μM HD, panel D, 300 μM HD 500 μM oxidized ATP, panel E, 400 μM HD 500 μM oxidized ATP Panel F shows the quantitation of the data and illustrates that oxidized ATP shifts the mode of cell death from apoptosis to necrosis Results represent the mean - - standard error of the mean of three experiments*

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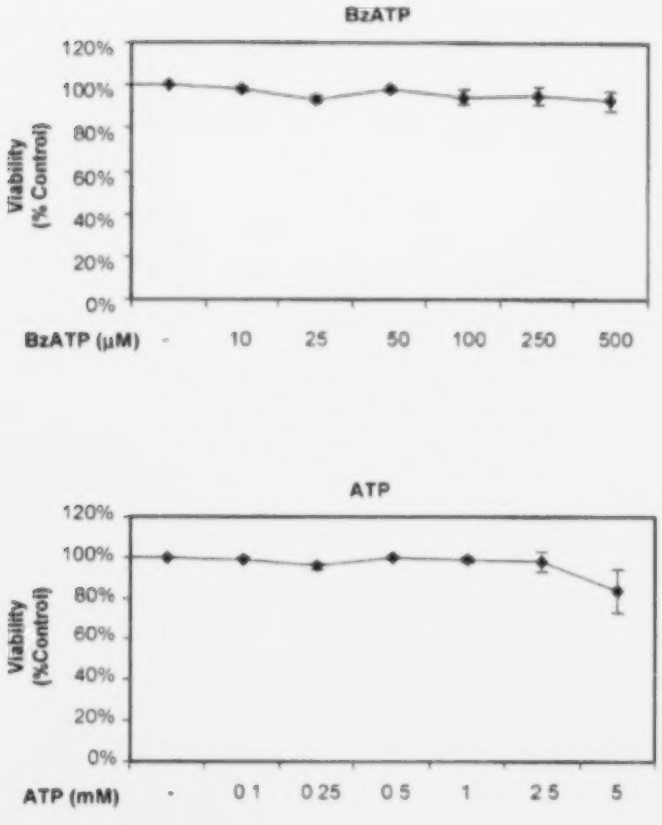

Figure 23. *Toxicity of ATP and BzATP in CHOK-1 cells Subconfluent cultures of CHO-K1 cells were treated with varying concentrations of BzATP (a) or ATP (b) for 24 h The viability of the cells was then assessed using the metabolic dye indicator alamarBlue No treatment caused significant cell death Results represent the mean ± standard error of the mean of three experiments*

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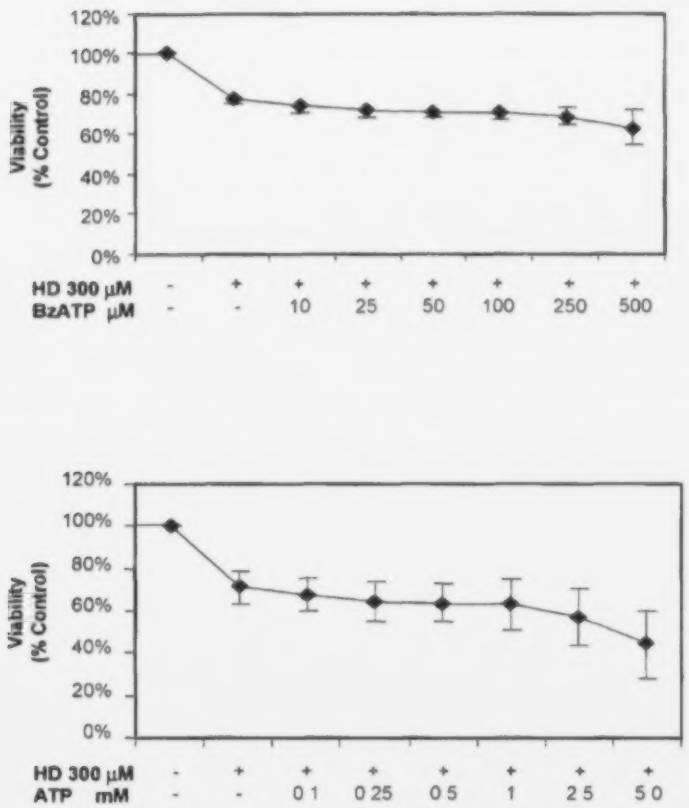

Figure 24. *Effect of ATP and BzATP on HD toxicity in CHO-K1 cells. Subconfluent cultures of CHO-K1 cells were treated with varying concentrations of BzATP (a) or ATP (b) overnight. The cultures were then treated with 300 µM HD for 24 h. The viability of the cells was then assessed using the metabolic dye indicator alamarBlue. Neither ATP nor BzATP significantly modified HD induced cell death. Results represent the mean ± standard error of the mean of three experiments.*

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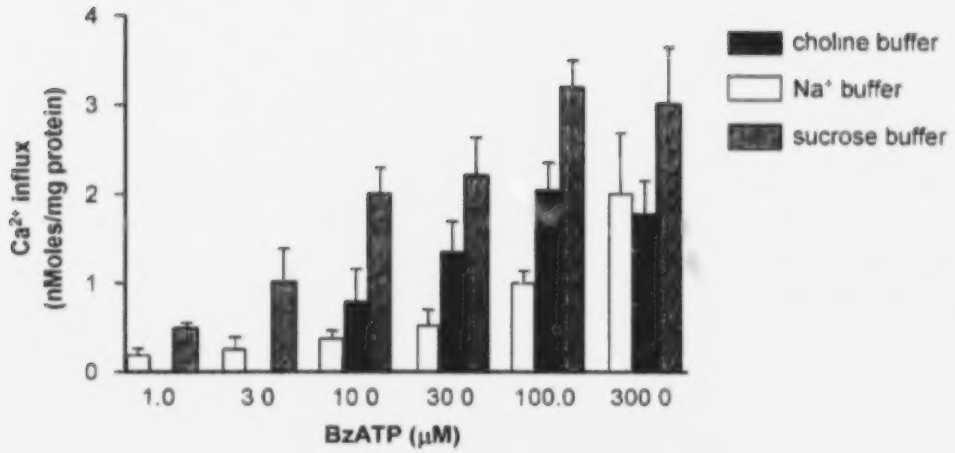

Figure 25. *Calcium influx induced by P2X-agonists in synaptosomes. Calcium influx was carried out in synaptosomal preparations exposed to increasing concentrations of BzATP in three buffer solutions. The rank order of calcium influx stimulated by BzATP in the three buffers was sucrose > choline chloride based buffer > Na chloride, a rank order which typifies the presence of P2X-receptors*

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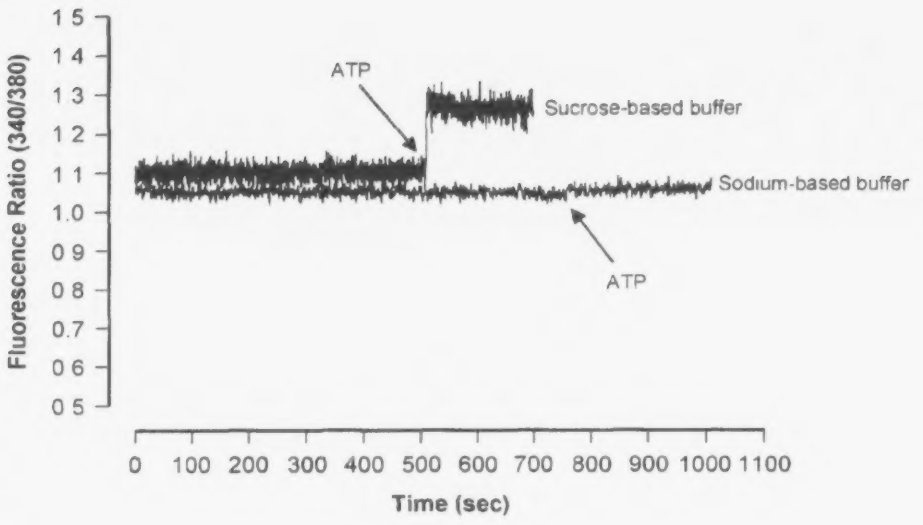

Figure 26. *ATP induced elevation of intracellular calcium in buffers of different ionic strength. Synaptosomes were incubated in either sodium chloride based buffer or in sucrose based buffer and the intracellular level of calcium measured following the addition of 1 0 mM ATP ATP was much more effective elevating calcium in the sucrose buffer than in the high ionic sodium buffer Results represent the mean + - standard error of the mean of three experiments*

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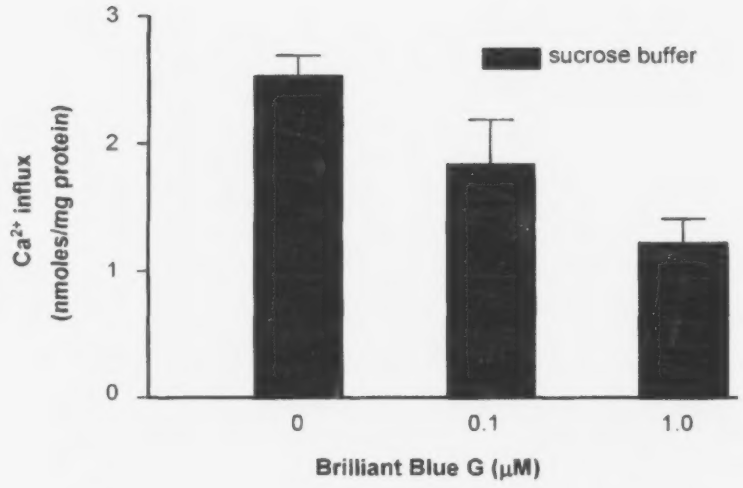

Figure 27. *Inhibition of BzATP induced calcium influx in buffers of different ionic strength BzATP induced calcium influx was measured in sucrose solution as previously described Influx was allowed to proceed for 60 sec in the presence of various concentrations of Brilliant Blue G, a selective P2X- antagonist BzATP induced influx was reduced in a concentration related fashion by the antagonist.*

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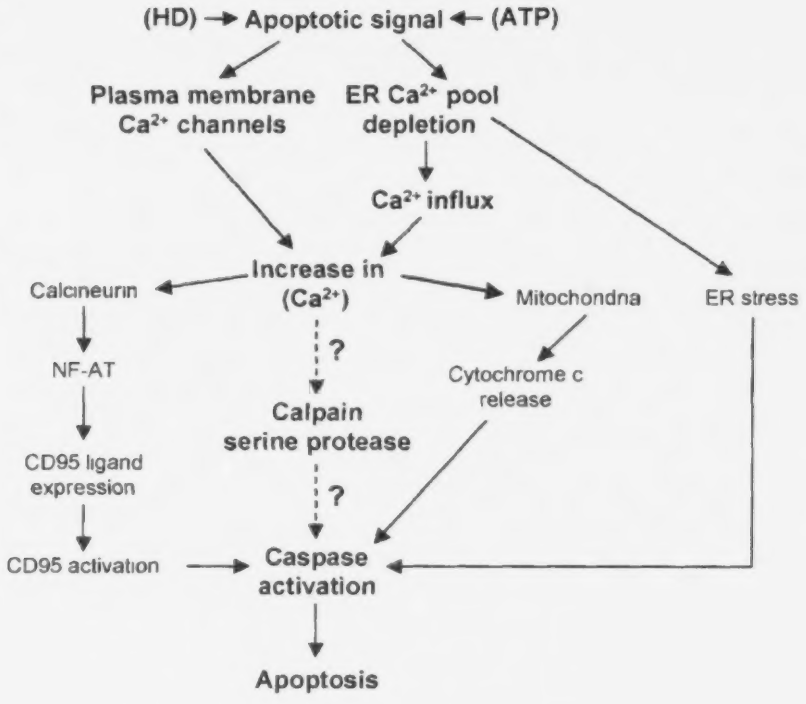

Figure 28. *This figure is a schematic representation of events thought to be important in the development of apoptosis (modified from Kass and Orrenius 1999) The highlighted portion in blue represents some of the efforts made in this laboratory to study possible mechanisms in the apoptosis caused by HD or ATP*

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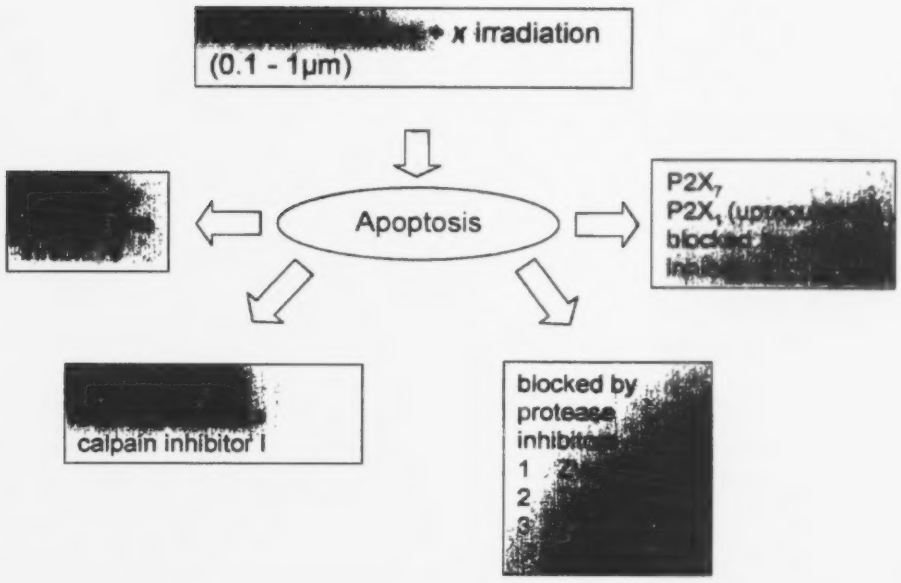

Figure 29. *Schematic diagram showing the relationship of the apoptotic inducers dexamethasone and X-irradiation with HD. Dexamethasone and X-irradiation both cause apoptosis which can be inhibited by proteasome inhibitors, calpain inhibitors and several protease inhibitors. The role of ATP receptors in the apoptosis induced by these agents is unknown with certainty but, dexamethasone upregulates the $P2X_7$ receptor in certain cells. The text of this report clearly shows that HD induced apoptosis is largely unaffected by these treatments.*

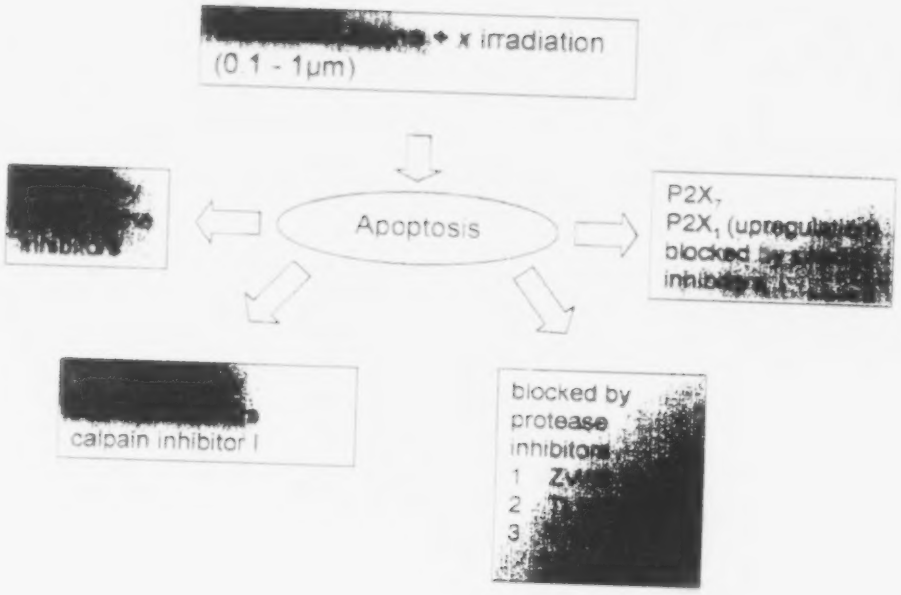

Figure 29. Schematic diagram showing the relationships of the apoptotic inducers dexamethasone and X-irradiation with HF1. Dexamethasone and X-irradiation both cause apoptosis which can be inhibited by proteasome inhibitors, calpain inhibitors and several protease inhibitors. The role of ATP receptors in the apoptosis induced by these agents is unknown with certainty but, dexamethasone upregulates the P2X7 receptor in certain cells. The data of this report clearly shows that HF1-induced apoptosis is largely unaffected by these treatments.

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A possible mechanism of action of sulphur mustard

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HD caused apoptosis and to a lesser degree, necrosis in a wide variety of cell types. As the concentration of HD was increased the proportion of necrotic cells became more predominant HD produced a modest elevation of intracellular calcium levels in J774 and CHOK-1 cells, as well as in human skin keratinocytes, although this rise did not seem causally related to HD induced cell death. However, very recent evidence indicates that HD induced DNA fragmentation may not be directly related to cytotoxicity HD also activated caspase-3 in CHOK-1 cells, but neither specific nor general caspase inhibitors nor proteosome inhibitors reduced HD cytotoxicity. These results suggest that the toxicity of HD is fundamentally different than apoptotic stimulae such as dexamethasone or X-irradiation, in which the apoptosis induced by both of these treatments can be reduced by the above mentioned protease inhibitors. Subtypes of the P2X receptors were identified in neuronal synaptosomes, J774 cells and CHOK-1 cells. In CHOK-1 cells $P2X_7$ receptor subtypes were identified and the specific $P2X_7$ inhibitor, oxidized ATP, was found effective in reducing HD induced DNA fragmentation and the appearance of soluble DNA. However, this treatment appeared only to shunt the mechanism of cell death from being predominantly apoptotic, to more necrotic in nature; however, the overall cytotoxicity remained unchanged.

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